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Pre-clinical Assessment of Recovery after Stroke: Factors Affecting Outcome

by

Suzanne B. DeBow

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Centre for Neuroscience

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Dedication:

This thesis is dedicated to my parents, Shirley and Gerald, for their infinite love and support. In addition, I would not have begun this journey without the inspiration and encouragement I received from my mentor, Dr. Bryan Kolb at the University of Lethbridge.

Abstract

Animal research has, and will undoubtedly continue to improve outcome for stroke survivors. However, controversy exists regarding the effectiveness of animal models and whether they are used properly. A survey of current ischemia studies was done to discern whether methodological procedures are improving (chapter 2). We found many rodent studies examining the efficacy of cytoprotective agents (e.g. cell saving) are lacking in many aspects (e.g., a predominance of studies assessed outcome in young male animals). One important implication is the lack of proper temperature measurement and control. Accordingly, a new method of temperature measurement that allows for continual recording via telemetry in the conscious animal is presented (chapter 3). Subsequently, two chapters examined the vulnerability of hippocampal CA1 neurons to secondary stressors (e.g., stress induced fever (SIF) due to rectal probe measurement; secondary sub-lethal transient ischemic attacks) after global ischemia in the gerbil. Rectal probe measurement causes a profound SIF that differs in ischemic and normal gerbils. Moreover, ischemic neurons salvaged by cytoprotection are susceptible to secondary ischemic insults (chapters 3 and 4 respectively).

Finally, the effects of rehabilitation after stroke were examined in two animal models of stroke. Constraint-induced movement therapy (CIMT) is used to encourage motor recovery of the hemiplegic limb in stroke patients (via immobilization of the good limb), and provides persistent benefit even when applied months after injury. However, CIMT *exacerbates* brain damage and reduces functional recovery in the rodent, contradicting clinical results. Post-ischemic hyperthermia potentiates ischemic injury, and was therefore examined as one potential factor in this CIMT-induced aggravation of brain

injury (chapter 6). Results show CIMT causes a significant increase in brain temperature in the tissue surrounding injury, suggesting hyperthermia may, at least in part, cause potentiation of injury due to CIMT. Finally, the effects of CIMT in a rodent model of intracerebral hemorrhage (ICH) were examined. For the first time after hemorrhagic stroke in the rat, rehabilitation provided both functional and neurological recovery.

In summary, these data show a critical period of neuronal vulnerability exists in the post-lesion period, and that neurons are susceptible to a number of factors during this time. Further, these data emphasize the importance of properly monitoring and controlling these factors (e.g., temperature fluctuations) during this period, particularly in the clinic.

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List of Abbreviations:

2-VO	Two vessel occlusion
4-VO	Four vessel occlusion
BCCAO	Bilateral common carotid artery occlusion
CIMT	Constraint induced movement therapy
CVD	Cerebral vascular disease
DND	Delayed Neuronal Death
EEG	Electroencephalogram
ICH	Intracerebral hemorrhage
MCA	Middle Cerebral Artery Occlusion
NMDA	*N*-methyl-D-aspartate
rt-PA	Recombinant tissue plasminogen activator
SHR	Spontaneously hypertensive rat
SIF	Stress induced fever
TIA	Transient ischemic attack

Chapter 1

Introduction

Each year between 40,000 and 50,000 Canadians suffer a cerebral vascular accident (CVA) from stroke, the third leading cause of death in the country, and the leading cause of morbidity. Of those who survive, 60% suffer permanent neurological and physical disabilities, as well as an increased chance (~20%) of having another event within two years (Heart and Stroke Foundation, 2003).

Classification of Stroke

Cerebral vascular accidents result when blood supply to the brain is suddenly interrupted, and can be divided into ischemic or hemorrhagic injury. Cerebral ischemia, which accounts for 80-85% of all strokes, is an interruption of blood supply to a region of the brain due to an occluded vessel (e.g., focal ischemia) or cardiac arrest (e.g., global ischemia; Table 1). The majority of ischemic insults (50-60% of all cases) result from: 1) large or small vessel thrombosis, 2) atheroemolism (clot originating from a diseased artery), 3) cardioembolism (clot originated in the heart), or are 4) "lacunar", due to small vessel disease affecting the circle of Willis. For instance, middle cerebral artery occlusion, which is one of the most common types of stroke, results from cardioembolic origins (Lhermitte et al., 1970). Extracranial atherosclerotic vascular disease, primarily of the carotid artery bifurcation, accounts for about 20% of ischemic strokes, while 30-40% of ischemic stroke remains unexplained (Elkind, 2003).

Hemorrhagic stroke, which accounts for 15-20% of all cases, occurs when a blood vessel in the brain bursts, spilling blood into or around the brain. Subarachnoid hemorrhage is uncontrolled bleeding in the subarachnoid space, and is often caused by closed head injury or the rupture of an aneurysm. Intracerebral hemorrhage results when blood vessels within the brain tissue rupture, often as a result of hypertension (Elkind,

2003). Although less common, hemorrhagic stroke is considered to be much more severe as 50% of patients die within 30 days.

Prevalence and Risk Factors

Despite advancing medical (e.g., surgical) treatments for heart disease, the incidence of CVA continues to rise due to a number of factors ranging from a poor choice of lifestyle to the ageing population. Between 1991 and 2001, the number of Canadians aged 80 years and over rose by 41% to 932,000 and is expected to increase an additional 43% in the next ten years. By then, it will have surpassed an estimated 1.3 million people (Heart and Stroke Foundation, 2003). Costs of treatments, rehabilitation and home care are expensive and will only continue to raise the economic burden of stroke. In addition to the financial burden, stroke causes and overwhelming emotional burden for families of survivors and the victims themselves. The testing of new strategies to motivate lifestyle changes, such as smoking cessation or the promotion of increased physical activity, should lead to more widespread awareness and prevention. Unfortunately, some risk factors for stroke are inherent and cannot be prevented, while modifiable risk factors cannot always be regulated. Thus, research examining new pharmacological agents and innovative surgical approaches are critical if we are ever to lessen morbidity and improve quality of life for survivors.

Eight in ten Canadians have at least one risk factor for cerebral vascular disease (CVD), while 11% have 3 risk factors or more (Heart and Stroke Foundation, 2003). Some risk factors are controllable while others are not. Controllable factors include smoking, alcohol abuse, physical inactivity, poor nutrition, obesity, raised blood pressure and blood glucose (Heart and Stroke Foundation, 2003). Uncontrollable risk factors

include diabetes, age, race, gender and a family history of heart disease. Prevention, or controlling risk factors, is only one of three main approaches to stroke treatment. While stroke continues to occur, post-stroke therapies initiated immediately after injury (acute) and long-term care (chronic) will remain equally important. In addition, recent attempts to find pharmacological treatment for acute stroke have focused on modulation of the molecular effects of stroke on the brain (Wahlgren, 1997).

Deficits

Cerebral ischemia is divided into two categories, 1) *global*: the loss of blood flow to the *entire* brain (e.g., cardiac arrest), and 2) *focal*: the loss of blood flow to a *particular region* of the brain (e.g. stroke). In both categories, rapid onset of neurological deficits occurs. Following focal ischemia, numbness of the face (e.g., aphasia), arm or leg (e.g., hemiparesis), sudden confusion, sudden visual difficulties, difficulty walking, dizziness or loss of balance and coordination and severe headache are often observed depending on the location of artery occlusion (Hemphill, 1998). Global ischemia also results in sudden confusion, dizziness and sometimes permanent memory loss. Clinicians diagnose the type of stroke using both physical examinations of the patient's symptoms in combination with various neuroimaging techniques. Stroke diagnosis can be straightforward when the symptoms are typical. Often however, stroke syndromes are less obvious and diagnosis may be delayed by hours, days or even weeks. Longer delays in diagnosis ultimately lead to longer delays in therapeutic interventions resulting in a decreased chance of functional and neurological recovery.

Functional Recovery after Stroke

Recovery from CVA depends on several factors. The size (i.e., severity) and location of brain damage, the patients general health (i.e., presence of diabetes, hypertension) and the medical care received (e.g., traditional hospital ward vs. specialized stroke wards). Many survivors of brain injury show spontaneous recovery to some extent (Kwakkel et al., 1997) without any form of intervention. Unfortunately, the majority of stroke patients do not show this remarkable recovery and they are left with chronic motor impairments. For instance, only 4% of patients with severe recovered independent function. (Jorgensen et al., 1999) Thus, recovery from stroke-related impairments depends upon the type of rehabilitation received. Functional recovery is significantly greater when patients receive individualized care on a stroke rehabilitation ward (Kalra, 1994; Glader et al., 2001; Evans et al., 2002), since treatment is administered early after stroke, reducing secondary complications and increasing long-term functional recovery (Hamrin, 1982). Stroke units are beneficial because specific types of rehabilitation are applied to meet individual patients needs (Ostendorf and Wolf, 1981; Wolf et al., 1989; van der Lee et al., 1999b). Currently, general approaches to rehabilitation include: 1) prevention of secondary complications that may occur post-injury; 2) reduction of neurological deficits; and 3) maintenance of behavioural/motor function over the long term (Gresham et al., 1995).

One of the most common disabilities resulting from stroke is paralysis of the body contralateral to the brain damage (hemiplegia). Following injury, patients typically learn to compensate with their good limb(s) and stop using their impaired limb(s) for daily tasks (i.e. increased dependence on the normal side), a phenomenon known as "learned-

non use" (Taub, 1980). Traditional rehabilitation promotes functional recovery by engaging the patient in physical exercises, beginning soon after stroke, that promote compensatory strategies with the good limb (Gresham et al., 1995). Those patients showing the greatest improvement do not have any other major medical illnesses, sensory or visual impairments, mental disorders or language problems impairing recovery (Ernst, 1990), but are still left with deficits on the impaired side of the body. More recently, concepts in rehabilitation strategies have involved treatments such as constraint-induced movement therapy (CIMT), that focus specifically on overcoming deficits of the impaired limb (i.e., learned-non use; Ostendorf and Wolf, 1981; Wolf et al., 1989). For instance, patients undergoing CIMT have their good arm restrained in a hand splint or arm sling encouraging the use of their impaired arm (Taub et al., 1993). To date, studies assessing CIMT therapy show lasting benefits even when applied a year after injury (Ostendorf and Wolf, 1981; Wolf et al., 1989; Taub et al., 1993; Miltner et al., 1999; van der Lee et al., 1999a). Thus, specific therapy for stroke patients directed at improving hemiplegia provide lasting, and should become common practice in the clinic. Unfortunately, therapies focusing on the impaired limb after stroke are still considered experimental.

Neurological Treatment after Stroke

Severity and duration of ischemic episodes profoundly affect outcome. Many acute neuroprotective strategies (e.g., cytoprotective agents, clot dissolving agents) are thus focused on both reducing the duration of injury and reducing metabolic disturbances initiated during injury. Recently, numerous neuroprotective agents have been developed in an attempt to decrease brain damage (Ovbiagele et al., 2003). Animal stroke models have yielded overwhelmingly promising results. For instance, drugs such as competitive

and non-competitive calcium, sodium and glutamate channel antagonists; drugs acting on free radical and nitric oxide related toxicity; apoptosis inhibitors and $GABA_A$ receptor agonists are beneficial in rodents (for reviews see Dyker and Lees, 1996; Lees, 2000, 2002). However, the majority of phase III clinical trials assessing neuroprotective agents conducted to date have failed. Despite promising experimental results, recombinant tissue plasminogen activator (rt-PA) given within 3 hours of the ischemic episode is the only treatment for stroke in use with proven clinical efficacy (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995). Unfortunately, due to the short critical period in which the drug improves outcome, only ~ 10 % of stroke survivors benefit from treatment, leaving the majority of untreated patients with debilitating chronic motor impairments.

Animal Models

Many experimental models of human cerebral injury are used to study mechanisms of cell damage and death, and to test novel therapeutic agents. Although no one model exactly mimics all of the clinical conditions of stroke, they allow for a number of factors, including severity and location of injury, age of the animal, and type of insult to be manipulated (Garcia, 1984; Ginsberg and Busto, 1989; Kim, 1997). Rodent models of stroke are by far the most common, although larger mammals (e.g., non-human primates, dogs, and cats), as well as non-mammalian vertebrates, have also been used. In addition to these *in vivo* models, many common *in vitro* models (e.g., cell cultures) exist, that are used to identify cellular mechanisms of injury and to screen putative neuroprotectants.

Typically, models of global and focal ischemia involve occluding arterial blood flow to the brain. Hemorrhagic models involve intracranial infusions of either the animal's blood to mimic the rupture of the artery wall, or enzymes that will degrade the artery wall itself (Rosenberg et al., 1990; Del Bigio et al., 1996). Most ischemic injury is induced while the animal is under anesthetic while physiological variables are closely monitored. For instance, brain temperature during injury clearly alters outcome following ischemia, and must be strictly maintained (Busto et al., 1989; Colbourne et al., 1997). Common rodent models used to induce global, focal and hemorrhagic stroke are summarized below.

Global ischemia

Rodent models of global ischemia are an attempt to mimic the pathophysiology of human cardiac arrest. Permanent global ischemia results in death, whereas transient global ischemia in gerbils (Kirino, 1982) and rats (Pulsinelli et al., 1982) results in patterns of selective neuronal vulnerability similar to that found in humans (Petito et al., 1987). Interruption of blood flow to the entire brain triggers hippocampal CA1 neuronal cell death and injury to the dorsolateral striatum (Kirino, 1982; Pulsinelli et al., 1982; Petito et al., 1987; Colbourne et al., 1999b). More severe insults also result in cortical damage (Kirino, 1982; Pulsinelli et al., 1982). Surprisingly, some hippocampal neurons survive the ischemic insult and show only subtle morphological changes for the first 24-hours following the insult (Kirino, 1982). These neurons eventually die several days later, a process known as *delayed neuronal death* (DND). A substantial delay (e.g., 2-4 days) exists between the end of ischemia and complete loss of hippocampal neurons, the duration of which appears to depend on the severity of injury. For instance, CA 1 cell

death was not observed 24 hours following injury but progressed slowly 3, 7, and 14 days, with 24%, 52%, and 59% percent of cells lost respectively (Colbourne et al., 1999a). This "delayed neuronal death" (Kirino, 1982), provides researchers with a potential window of opportunity to employ therapeutic interventions (Choi, 1985).

- **Two-Vessel Occlusion in the Rat**

The two-vessel occlusion model (2VO) results in reversible forebrain ischemia and is produced by bilateral common carotid artery occlusion combined with systemic hypotension (~ 50 mmHg; Smith et al., 1984a) to prevent flow through collateral arteries. Blood flow drops to less than 5 % of controls in the cerebral cortex and less than 1 % in the hippocampus and striatum (Smith et al., 1984a; Smith et al., 1984b). Differences in reduction of blood flow to various regions of the brain may, at least in part, explain the selective neuronal death observed following global ischemia. Within 15 - 25 seconds of ischemia, the electroencephalogram (EEG) becomes isoelectric (Smith et al., 1984a). This model results in selective cell death in the CA1 region of the hippocampus and the maturation of cell death after injury depends greatly upon the severity, and duration (10 - 30 minutes), of ischemia. One advantage of this model is the one-stage surgical preparation.

- **Four-Vessel Occlusion in the Rat**

The four-vessel occlusion model (4VO), includes two surgical preparations in the same animal (Pulsinelli et al., 1982). The first involves electrocauterization of the vertebral arteries to prevent compensation of blood flow through the posterior communicating arteries during the ischemic insult. Due to the circle of Willis this stop alone does not cause deleterious effects. The second stage occurs twenty-four hours later,

when both common carotid arteries are temporarily occluded with small arterial clamps, producing high-grade forebrain ischemia. At the end of the ischemia, the clamps are removed and circulation is restored. The 4VO model is known to be highly reproducible (Ginsberg and Busto, 1989), decreasing blood to ~ 3 % of control animals in the cortex and the striatum, and between 3 - 7% in the hippocampus (Pulsinelli et al., 1982). The EEG typically becomes isoelectric within 8-10 seconds (Zagrean et al., 1995), and spontaneous cortical activity is abolished within 1 minute (Xu and Pulsinelli, 1994). One advantage of this model is that it may be used without anesthetic, which some would argue is more relevant to human injury since humans are rarely under anesthesia when they fall victim to stroke. Despite this, most researchers prefer to use some anesthesia, particularly during the carotid artery occlusion.

- **Bilateral common carotid artery occlusion in the gerbil**

Forebrain ischemia in the gerbil is produced by temporarily clamping both common carotid arteries. Gerbils have an incomplete circle of Willis with no posterior communicating arteries (Levy and Brierley, 1974). Therefore, simply clamping the carotids results in complete forebrain ischemia (Kirino, 1982; Kirino et al., 1985). Selective cell death of CA1 neurons occurs, similar to global ischemia models in the rat, as blood flow drops to less than 1 % in the cortex, and ~ 4 % of controls (Kato et al., 1992). Electroencephalogram failure also occurs quickly (within 20 seconds; Suzuki, 1983).

Rodent models of ischemia using vessel occlusion are often deemed "incomplete" because of residual blood flow through collateral arterial systems. Models of "complete ischemia" attempt to overcome this by preventing blood flow to the entire brain. Blood

flow is zero or less than 1% of controls over the entire brain in these models. For instance, a neck-cuff is used (Ljunggren et al., 1974), or compressing all arteries stemming from the heart (Kawai et al., 1992). Due to severe mortality in these models, they are not commonly used.

Focal ischemia

Focal ischemia in the rodent typically involves occlusion of the middle cerebral artery (MCA). These models have gained increased acceptance due to their clinical relevance, as the MCA is the most commonly occluded vessel in human stroke victims (Ginsberg and Busto, 1989; Karpiak et al., 1989). Variations of the MCA model can be classified as either permanent or transient. In both permanent and models, there is a *core* ischemic region (blood flow is reduced to < 10%) which is the region of brain primarily supplied by the occluded artery. Additionally, there is a *penumbral* region surrounding the ischemic core where blood supply remains at sufficient levels to maintain cell survival (blood flow is reduced < 40%; Tamura et al., 1981; Nedergaard et al., 1986; Tasker et al., 1996; Tateishi et al., 1998).

- **Middle Cerebral Artery Occlusion**

In permanent focal ischemia, the arterial blockage remains throughout the entire experiment, resulting in severe reductions in blood flow to the injury core. Initially, this model consisted of cauterizing the MCA proximal to the origin of the lateral lenticulostriate arteries, thereby preventing collateral blood flow from those arteries (Tamura et al., 1981). More recently, sutures or clips are used to clamp the MCA both distal and proximal to the lenticulostriate branches (Longa et al., 1989; Buchan et al., 1992). Although the protocol differs somewhat across labs, the suture model involves

inserting a nylon filament through the external carotid artery and into the internal carotid artery occluding the MCA at its origin (Longa et al., 1989). Advantages of this model include the fact that no craniotomy is required, allowing normal increases in intracerebral pressure to occur. The MCA model also has the advantage that it can be used as a permanent or transient model. For this reason, the suture model is arguably the most clinically relevant, although difficulties with insult variability remain to be resolved. Further, arterial damage may occur following suture insertion and removal resulting in poor reperfusion of brain tissue and large infarcts.

In all focal MCA models, post-ischemic temperature monitoring is crucial, particularly following the suture model, as proximal blockage of the MCA can result in hypothalamic damage preventing animals from regulating their own temperature. In an attempt to overcome extensive hypothalamic and basal ganglia damage, distal occlusion of the MCA can also be done with the clip model. However, this model requires a craniotomy, and often the rats' zygomatic arch is broken, resulting in relatively adverse surgical process. Further, distally occluding the MCA requires occlusion of the ipsilateral common carotid artery for adequate reduction of blood flow.

As previously mentioned, hypertension is one of many risk factors for stroke. As such, spontaneously hypertensive rats (SHRs), which are more susceptible to ischemic injury are commonly used instead of normotensive rats. For instance, MCA occlusion results in larger and more consistent cortical infarcts in SHRs than in normotensive strains (Coyle, 1986; Coyle and Heistad, 1986, 1991; Kita et al., 1995).

- **Thromboembolism**

In the clinic, stroke is commonly due to the formation of thrombus initiating the ischemic episode. Thus, many models of thrombosis exist in the rodent. One type of thrombotic model consists of using a photosensitive dye perfused through a localized arterial system and laser irradiation to induce endothelial damage and platelet aggregation (Watson et al., 1985). Autoradiographic studies show this procedure results in small vessel thrombosis in predetermined cortical regions (Dietrich et al., 1989). Dye-light reactions cause arterial damage that leads to increased blood-brain barrier permeability, platelet recruitment and activation in arteries supplying a region of brain designated by placement of the laser on the skull (Dietrich et al., 1989; Dietrich et al., 1994). Unfortunately, large variability in blood flow reductions, as well as infarct size, necessitates large numbers of animals to obtain statistical significance, particularly for drug testing. In addition, the exact location of the clot in the artery is not easily defined.

Thrombin can also be infused directly into the internal carotid artery via an intraluminal catheter, resulting in a thrombus at the origin of the MCA (Zhang et al., 1997). Typically, this model is used to test various thrombolysis techniques, such as rt-PA (Meden et al., 1994; Overgaard et al., 1994).

Hemorrhage

Several intracranial hemorrhagic stroke models are used to investigate factors affecting injury size and functional recovery. As such, models attempt to mimic not only the toxic effects of blood leaking into the brain, but also the direct damage caused by the resulting hematoma. Bacterial collagenase, when infused into the brain tissue, results in the digestion of collagen within the arterial wall causing it to rupture and bleed into the surrounding parenchyma (Terai et al., 2003). Intrastriatal injection of bacterial

collagenase is commonly used to induce hemorrhagic stroke (Rosenthal and Fain, 1971). Heparin can also be added to the injection, resulting in less collagenase required to produce injury and functional impairment mimicking human damage (Del Bigio et al., 1996).

Another common hemorrhagic stroke model involves injection of autologous whole blood (or blood components such as thrombin or hemoglobin; Ropper and Zervas, 1982). This model is typically used in studies assessing temporal profile of cell death, edema formation, inflammatory response and disruption of the blood-brain barrier (Xue et al., 2000).

One final model of hemorrhagic stroke, which is much less common, is the insertion of an inflatable micro-balloon into the brain tissue. This model is used to characterize the effect of mass lesions, the progression of apoptosis, and reductions in cerebral blood flow after transient lesions (Nehls et al., 1988). However, due to lack of clinical relevance, this model is rarely used to assess neuroprotective agents or functional recovery.

Development and measurement of damage

Global Ischemia

During global ischemia, the loss of CBF results in energy failure in the cells leading to disruption of ionic gradients (Hansen, 1985). Specifically, the opening of voltage-dependent K^+ channels results in K^+ efflux from neurons (Choi, 1988; Choi, 1992; Lee et al., 2000). This efflux of K^+ results in membrane hyperpolarization, followed by abrupt membrane depolarization, influx of sodium, calcium and chloride ions causing the cell to begin to swell. The neuron swells due to osmostic movement of

water across the membrane as a result of the sodium and chloride entering the cell (Rothman and Samaie, 1985). Due to the lack of blood flow (i.e., oxygen supply to the cells) membrane depolarization is termed "anoxic" and results in the excessive release of neurotransmitters, in particular, glutamate, promoting further spatial spread of cellular depolarization (Choi, 1988; Choi, 1992; Lee et al., 2000). Glutamate-induced neuronal death, known as *excitotoxicity*, results in further depletion of energy stores and advancement of injury cascades. As such, many neuroprotective strategies (e.g., NMDA antagonists) have attempted to block excessive release of glutamate.

Although glutamate is thought to play an important role in cell death following ischemia, the presence of extracellular calcium influx into the cell is also an important factor in ischemia-induced neuronal death (Choi, 1992). Following injury, intracellular calcium levels slowly increase to toxic levels, due to impaired secondary calcium cascades, the loss of membrane homeostasis and/or loss of intracellular calcium regulation (Dienel, 1984; Deshpande et al., 1987; Andine et al., 1992; Ohta et al., 1992). When these toxic levels of intracellular calcium persist, mitochondria inside the cell become overloaded causing the production of free radicals, as well as irreversible damage to membranes. Therefore, excessive glutamate release during and following ischemia damages cells, at least in part via calcium-dependent processes, and ultimately causes cell death. Given that cell death following global ischemia occurs over 2-3 days, therapeutic intervention early after injury during the critical period before neuronal death could potentially rescue vulnerable cells. Therefore, delayed death and the mechanisms underlying excitotoxicity-induced cell death provide a window of opportunity for cytoprotective therapies.

Focal Ischemia

Cell death develops in two ways following focal ischemia. The first is a central *core* region, and the *penumbra* surrounding it (Hossmann, 1994). After short (e.g., 30 minute) focal ischemia, cortical damage is not visible immediately, but matures over 3 days following the insult (Du et al., 1996). However, after longer focal ischemia (e.g., 90 minute), cortical damage is evident within 6 hours and completely developed within 24 hours (Du et al., 1996). Cell death in both brief and lengthy ischemia occurs despite the recovery of blood flow, energy metabolism and electrical activity (Du et al., 1996). This suggests that the delayed cell death phenomenon observed following global ischemia also occurs after focal ischemia.

Summary

Overwhelming scientific knowledge about stroke is currently available, providing the possibility of stroke prevention within the near future. However, as cerebral vascular accidents continue to occur, strategies focusing on promoting rapid recovery and independent living for the stroke patient are still needed. Accordingly, studies examining the effectiveness of rehabilitation strategies, as well as neuroprotective agents are abundant. The objective of this thesis was to explore neurological (e.g., histological) and functional (e.g., behavioural) recovery after stroke. Specifically, the first two chapters address recent controversies into the effectiveness of animal models of human stroke (chapters 2 and 3). In the first article, a survey of current studies examining cytoprotective agents was done to characterize recent ischemia studies, and to discern whether these studies are improving methodological procedures. In the second article, current methods and techniques of temperature measurement are reviewed. Primarily, a

new method of temperature measurement in the conscious animal is discussed, since it is the most effective for continual recording and maintenance of both body and brain temperature.

The third and fourth chapters of this thesis deal with global ischemia in the gerbil. In this model, intra- and postischemic hypothermia improves outcome, whereas hyperthermia worsens it. Many studies use rectal probe measurements as a means to determine postischemic temperature changes. However, the probe procedure itself can alter temperature since it causes a stress-induced fever (SIF). Therefore, the effects of secondary injury due to post-ischemic SIF in the gerbil were examined to characterize its effects in both the ischemic and non-ischemic gerbil, as well as to discern if the SIF would worsen histological outcome (chapter 4). In the fifth chapter, the effect of secondary injury on neurons previously protected with postischemic hypothermia was assessed. In this study, gerbils were treated with mild hypothermia beginning 12 hours after a 5-minute global ischemia and then subjected to a 1.5-minute transient ischemic attack (TIA) five and six days after surgery to determine the resiliency of hypothermia treatment.

Finally, the last two articles of this thesis examine the effects of rehabilitation strategies following both cortical and sub-cortical injury. Treatment of the upper extremity of stroke patients suffering from hemiplegia (e.g., CIMT) continues to be one of the most successful post-stroke therapeutic interventions. However, in rodent models of CIMT, immediate immobilization of the good limb following unilateral cortical lesions results in *exacerbation* of damage and reduced functional recovery, contradicting clinical results. Since post-ischemic temperature aggravates cerebral injury, the sixth chapter of

this thesis was done to determine the effects of CIMT on both core and brain temperature with telemetry probes following unilateral devascularization lesions of the motor cortex. Finally, CIMT 8 h/d over 7 d in combination with a relatively mild-moderate exercise regimen (e.g., running wheel, horizontal ladder and skilled reaching) was examined in a rodent model of intracerebral hemorrhage (ICH). Overall, this thesis examines a wide range of factors affecting recovery after stroke, such as post-ischemic temperature changes and rehabilitation strategies. It is hoped that knowledge gained from these studies will aid future research in the treatment of stroke.

Table 1.1

This table lists the two main types of cerebral vascular accident (i.e., ischemic or hemorrhagic), the animal model used to mimic each type of injury, the percentage of efficacy studies surveyed in Chapter 2 examining each rodent model and the percent of population presenting with each type of stroke clinically. Note: percentages of efficacy studies do not include results from mouse studies (See Table 2.1) and thus do not equal 100%. See text for a more detailed description of the subdivisions of each category and animal models.

Type of Stroke	Animal Model	Percentage of ischemia efficacy studies	Proportion of all Strokes in humans
Ischemia			**80-85 %**
• Global	• Two-vessel occlusion (rat)	• 15%	
	• Four-vessel occlusion (rat)	• 20%	
	• Common carotid occlusion (gerbil mouse)	• 50%	
• Focal	• Middle cerebral artery occlusion (cautery, clip or embolism)	• 22.9%	
	• Intraluminal suture model	• 50%	
Hemorrhage			**15-20 %**
• Subarachnoid	• Whole blood infusion	• 0%	
• Intracerebral	• Bacterial collagenase, thrombin or whole blood infusion, vessel rupture (due to puncture by intraluminal suture)	• 0%	

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Chapter 2

Incomplete Assessment of Experimental Cytoprotectants in Rodent Ischemia Studies

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Animal research has and will undoubtedly continue to improve outcome for humans who suffer from cerebral ischemia (e.g., stroke). However, the hope for an effective cytoprotective (cell saving) drug has given way to pessimism as these compounds have repeatedly failed clinical scrutiny (Gladstone et al., 2002). A number of likely explanations have been proposed (Corbett and Nurse, 1998; STAIR, 1999; DeGraba and Pettigrew, 2000; Gladstone et al., 2002). For instance, in many clinical trials the putative cytoprotectant was given after an unrealistically long intervention delay. At such long delays those compounds also failed pre-clinical testing (i.e., rodent studies). Thus, clinical trials were not always accurately based upon pre-clinical studies.

Inadequate pre-clinical assessment is also partly to blame for the premature advancement of some therapies to the clinic (Corbett and Nurse, 1998; STAIR, 1999; DeGraba and Pettigrew, 2000; Gladstone et al., 2002). For instance, the STAIR report (STAIR, 1999) recommended that putative cytoprotectants be tested in larger gyrencephalic species prior to clinical trials. However, efficacy has been and is based largely upon rodent ischemia models. One major limitation of many early studies has been the failure to assess long-term histological and functional outcome. Many studies in the 1980's and 1990's used survival times of 1 – 2 days for focal ischemia and 4 – 7 days for global ischemia, which are sufficient to allow the majority of cell death to mature after moderate to severe ischemic insults (Kirino, 1982; Pulsinelli et al., 1982; Garcia et al., 1993; Colbourne et al., 1999a). However, substantial cell death may evolve beyond these survival times when the insult is mild (Du et al., 1996; Garcia et al., 1997; Colbourne et al., 1999a) or treated with a cytoprotectant (Dietrich et al., 1993; Valtysson et al., 1994; Colbourne and Corbett, 1995; Wang et al., 2002). For example, Dietrich et

al. showed that three hours of postischemic hypothermia reduced CA1 cell death at three and seven days following global ischemia, but did not save CA1 neurons at two months (Dietrich et al., 1993).

In addition to using survival times that did not allow for injury to completely mature, many early studies either did not assess functional outcome or used simplistic neurological deficit scores and only in the first few days after stroke. Remarkably, long-term functional outcome is the clinical endpoint of greater concern, and yet most experimental studies relied upon quantification of early cell death, which does not necessarily correlate with eventual behavioural outcome (Wahl et al., 1992; Corbett and Nurse, 1998). Unfortunately, when behaviour was assessed, it was often done with a neurological deficit score (NDS) that not only varied among labs but was not able to precisely gauge longer-term deficits (Cregan et al., 1997) or distinguish true preservation of function from compensatory processes (Whishaw, 2000).

Stroke is more common in the elderly and yet the majority of early studies utilized young adult animals. Since there are outcome differences between young and old animals (Duverger and MacKenzie, 1988; Futrell et al., 1991; Sutherland et al., 1996) it seems appropriate to assess cytoprotectants in aged animals at some point. Furthermore, there are sex differences in ischemic injury and recovery (Roof and Hall, 2000) and yet this is ignored in most studies.

Finally, early rodent studies were plagued by temperature confounds. Ischemia and the use of an anesthetic (e.g., halothane, sodium pentobarbital) induce hypothermia, which is the best cytoprotectant. Some early studies failed to measure temperature during ischemia, yet aside from blood flow, temperature is arguably the most critical

determinant of ischemic injury (Dietrich et al., 1996; Colbourne et al., 1997). Furthermore, studies that assessed intra-ischemic temperature often relied upon rectal temperature measurements, which do not necessarily correlate well with brain temperature (Busto et al., 1987; Minamisawa et al., 1990; Colbourne et al., 1993). Accordingly, a drug may augment brain hypothermia even with core temperature maintained at normothermia. More recently, the importance of postischemic temperature has been recognized. Delayed fever substantially worsens global (Coimbra et al., 1996; Baena et al., 1997) and focal (Zhao et al., 1994; Memezawa et al., 1995; Kim et al., 1996; Reglodi et al., 2000) ischemic damage whereas delayed cooling reduces ischemic injury after global (Colbourne and Corbett, 1994; Colbourne and Corbett, 1995; Hickey et al., 2000) and focal cerebral ischemia (Colbourne et al., 2000; Corbett et al., 2000; Maier et al., 2001). For example, hypothermia delayed for 12 hours after global ischemia significantly reduced CA1 neuronal loss (Coimbra and Wieloch, 1994; Colbourne et al., 1999b). The consequence of not carefully assessing intra- and postischemic temperature is best illustrated by the findings with MK-801 (Buchan and Pulsinelli, 1990; Corbett et al., 1990) and NBQX (Nurse and Corbett, 1996) that reduced CA1 cell death largely or completely by inducing hypothermia.

The aforementioned limitations of experimental stroke studies have been also identified in literature reviews (Corbett and Nurse, 1998; STAIR, 1999; DeGraba and Pettigrew, 2000; Gladstone et al., 2002). In this study we surveyed all of the publications in *Experimental Neurology, Journal of Cerebral Blood Flow and Metabolism*, the *Journal of Neuroscience* and *Stroke* for the years of 2000, 2001 and 2002 combined, and this was compared to studies published in 1990 in the same journals. We identified those

ischemia studies that used adult to aged rodents to assess the efficacy of a cytoprotective treatment on histological and / or functional outcome. The purpose of this survey was to characterize recent ischemia studies and more importantly to determine whether these studies are plagued by methodological limitations.

Materials and Methods

Survey Scope

We identified all of the rodent ischemia articles published in the *Journal of Neuroscience*, the *Journal of Cerebral Blood Flow and Metabolism*, *Experimental Neurology* and *Stroke* for the years of 2000, 2001 and 2002 combined along with the year of 1990. Data were collapsed across the years 2000 to 2002 to improve the sample size of recent studies, which is the primary focus of this survey, and because we did not observe any notable change in practices during this time. In rare cases, two models and / or species were used in one publication. These were then considered to be separate studies. Only studies that used adult to aged rodents to assess global or focal ischemic insults were included; all others were excluded (e.g., neonatal hypoxia). We limited the survey to rodent models since these are, by far, the most commonly used to determine efficacy and mechanisms of action. Of these adult ischemia studies we included only those that tested a putative "cytoprotective" therapy (e.g., glutamate antagonist). A cytoprotection study was defined as one in which a therapy was administered (most common) or a genetic manipulation was made (e.g., knockout mouse; only a few studies) and the effects of that therapy were assessed on histological (including MRI determined lesion volumes) and/or behavioural outcome. Studies that *only* examined intra- and/or postischemic cellular alterations (e.g., caspase activation), without direct manipulation, were not

included. Studies that examined very late interventions (e.g., stem cell transplants one week after stroke) were not included.

A survey approach of four journals was chosen over a computerized literature search for several reasons including: 1) to limit the number of articles, 2) to ensure that each article included in the survey conformed to the above criteria, which required a detailed examination of each article, and 3) to avoid missing articles that did not use cytoprotection, neuroprotection or drug therapy, etc. as a subject heading, but were nonetheless efficacy studies.

Identification of Rodent Models

Studies surveyed used either rats, mice or gerbils. Global ischemia models in the rat included the two-vessel occlusion plus systemic hypotension (2-VO) and four-vessel occlusion (4-VO) models. In the gerbil and mouse the forebrain ischemia model used was bilateral common carotid artery occlusion (BCCAO). Focal ischemia studies used middle cerebral artery occlusion (MCAO) models either by intraluminal suture occlusion, clot occlusion (i.e., embolic model, photo thrombosis), electrocauterization or clip occlusion (or like procedure) of the MCA.

Identification of Species, Sex and Age

The species, sex and age of animals were identified. Many studies did not state the animals' age but instead stated a weight range. In these cases we categorized the age as undetermined since weight is not a reliable predictor of age and is also influenced by sex, health status, strain, etc. We assumed that these studies used young adults.

Identification of Temperature Measurement Technique

The methods used to measure intra- and post-surgery temperature were identified and categorized into: 1) rectal or core, 2) temporalis muscle or tympanic with or without measuring rectal / core temperature, and 3) brain temperature with or without other measurement methods. Post-surgery temperature measurement was defined as any measurement beyond the end of surgery and this was divided into the times of 0 – 2 hr, 2 – 6 hr, 6 – 12 hr and 12 – 24 hr (i.e., > 12 but 24 hr) after surgery. The sampling rate was categorized as: 1) continual (sampling at least every 15 minutes or continuously), 2) frequent (sampling every 16 – 60 minutes), 3) infrequent (sampling every 61 – 120 minutes), 4) rarely (< 1 sample every 2 hr) or 5) no postoperative sampling.

Survival Time and Choice of Endpoint

The survival time (hours to days following the start of ischemia) was categorized (e.g., 0 – 24 hr, 25 – 48 hr, etc.) for both global and focal ischemia studies. Behavioural evaluation was categorized as; absent, a NDS (e.g., a scale that includes ratings of spontaneous circling and activity, paw placement, etc.) or additional testing (e.g., skilled reaching, water maze testing, rotarod) with or without the NDS. Those studies that only used a behavioural evaluation procedure to determine whether animals were ischemic or not, but did not assess whether a treatment affected behavioural outcome, were not considered to have used behavioural assessments as an efficacy endpoint.

Results

According to our criteria of an efficacy study, we identified 19 and 20 global ischemia experiments for 1990 and 2000 – 2002, respectively. There were 6 and 118 focal ischemia experiments for 1990 and 2000 – 2002, respectively. The types of model,

sex and age characteristics are reported in Table 1. The vast majority of studies used male rodents and only two recent studies used old animals (> 1 year old). The intraluminal suture occlusion model has clearly replaced electrocautery as the preferred model of focal ischemia. Interestingly, almost all studies report positive results.

Survival time varied greatly for both focal and global ischemia studies (Figure 2.1). Eighty percent of recent global ischemia experiments used survival times of 7 days. Sixty six percent of recent focal ischemia studies used survival times of 48 hr whereas only 8.5% of focal ischemia studies examined histological outcome after 7 days. These results mirror those in 1990.

Out of 20 recent global ischemia studies only two assessed behaviour, which compares well to the one out of 19 studies that examined behaviour in 1990. In the recent focal ischemia studies, 55.1% did not assess functional outcome, 33.9% used some sort of NDS alone, and 11.0% used additional testing (e.g., skilled reaching) with or without a NDS. None of the 1990 studies used behavioural assessment as an endpoint.

The majority of global and focal ischemia studies used either rectal or core temperature measurements during ischemia without any other means of predicting brain temperature (Figure 2.2). Some studies utilized temporalis muscle (or skull) temperature. Very few studies directly measured brain temperature. Telemetry probes (core, brain, temporalis muscle) were rarely used in recent studies (15% of global ischemia studies; 2.5% of focal ischemia studies), and not at all in the 1990 papers we surveyed.

Post surgical temperature measurement after global ischemia, defined as any measurement beyond the end of anesthesia, which is likely < 10 minutes following the end of occlusion, occurred in 3 out of 19 and 6 out of 20 studies published in 1990 and

2000 – 2002, respectively. The three 1990 studies sampled rectal temperature intermittently for up to 2, 6 or 24 hr. Three of the 2000 – 2002 studies measured continually with telemetry probes for at least 24 hr. The other three studies sampled rectal temperature up to either 1 or 2 hours following ischemia. Several other studies stated that animals were placed in a room or a chamber with constant temperature for several hours after ischemia, but the animals' temperature was apparently not measured. The percentage of cytoprotection studies in focal ischemia that measured temperature following surgical anesthesia, even if once, was only 0% and 33.0% for 1990 and 2000 – 2002, respectively. Those 2000 – 2002 experiments that measured post-surgically were assessed for when they measured temperature and how often (see Methods, Figure 2.3). Only 3.4% of all 2000 – 2002 focal studies used a telemetry probe (e.g., core temperature). Most typically used intermittent rectal probe measurements. Sometimes animals were placed in temperature-controlled rooms without measuring the animals' temperatures.

Discussion

Numerous "cytoprotectants", which were beneficial in rodent ischemia studies, failed clinical trials. As reviewed (Corbett and Nurse, 1998; STAIR, 1999; DeGraba and Pettigrew, 2000; Gladstone et al., 2002), this inability to translate positive results from the bench to the bedside is due to incomplete experimental testing as well as limitations of clinical studies. Unfortunately, this survey of recent rodent ischemia studies demonstrates that most current experimental studies do not accurately represent clinical conditions of ischemia (e.g., aged animals) and many continue (cf. 1990 data) to have serious limitations that will likely contribute to further clinical failures.

Of greatest concern was the fact that 66% of focal and 80% of recent global ischemia cytoprotection studies used survival times of ≤ 48 hr and 7 days, respectively. These survival times, and even somewhat longer ones (e.g., 2 weeks), are not necessarily sufficient to allow injury to mature fully (Dietrich et al., 1993; Valtysson et al., 1994; Colbourne and Corbett, 1995; Du et al., 1996; Colbourne et al., 1999a; Wang et al., 2002). This is shown, for instance, in one of the papers surveyed where flavopiridol reduced CA1 cell death at a 7 but not a 28 day survival (Wang et al., 2002). Thus, the reduction in cell death observed in most experiments surveyed likely overestimates long-term benefit. The use of longer survival times (e.g., 1 month) is clearly required and usually easily achieved.

Only 45% of focal and 10% of global ischemia studies in 2000 – 2002 assessed behaviour. Thus, studies that did not assess behaviour may have overestimated benefit since not all reductions in cell death will translate into improved functional outcome. Conversely, behavioural improvements may occur without substantial reductions in cell death. Of those studies that assessed behaviour, most used only a NDS soon after injury (e.g., in first day). There are several potential problems with this approach. First, in some studies the animals were under the influence of the cytoprotectant at that time of behavioural testing. Second, the scales varied greatly (e.g., 2 – 10 point scales) and they were often not clearly described in the Methods, which makes it difficult to compare among studies. Third, a NDS, even the more comprehensive scales, may not necessarily predict long-term outcome. Indeed, deficits on these scales often resolve within a few weeks (Cregan et al., 1997). Only a few studies used another functional test (e.g., skilled reaching) that reveals more persistent behavioural abnormalities. Regardless, recovery on

either NDS or more sophisticated tests (e.g., staircase test) was never evaluated with respect to whether animals used compensatory strategies or whether they truly recovered (i.e., regained the original movement sequences in the test) (Whishaw, 2000). The latter would be expected to better predict clinical findings.

Most recent studies (82%) used male rodents. The inclusion of female animals is crucial, as approximately half of all ischemic insults occur in females. Only a very small number of the latest studies (1.5% overall) used aged animals. In order to efficiently and adequately evaluate potential cytoprotectants, studies demonstrating benefit in aged animals and both sexes are clearly required, at least at some point in the pre-clinical process. This does not appear to be happening.

Cerebral ischemic injury is critically dependent upon intra-ischemic temperature (Dietrich et al., 1996; Colbourne et al., 1997). Most studies assessed temperature during ischemia. Rectal temperature was most commonly used. However, while it roughly predicts brain temperature in conscious rodents (DeBow and Colbourne, 2003) this is not the case during global ischemia (Busto et al., 1987; Minamisawa et al., 1990; Colbourne et al., 1993). Accordingly, many of the global ischemia studies that used only rectal temperature measurements may be confounded by unknown variations in brain temperature. The relationship between brain and rectal temperature in focal ischemia is more complex and likely depends critically upon blood flow among other factors (e.g., craniotomy vs. intraluminal suture models, use of anesthesia, species).

Postischemic temperature can substantially modify focal ischemic brain damage. For example delayed hypothermia reduces infarct size (Zhao et al., 1994; Memezawa et al., 1995; Kim et al., 1996; Reglodi et al., 2000) and delayed hyperthermia aggravates

infarction (Colbourne et al., 2000; Corbett et al., 2000; Maier et al., 2001). Only a minority of global and focal ischemia studies measured post-surgical temperature even though all of the ischemia models have been shown to affect postoperative temperature (Zhao et al., 1994; Colbourne and Corbett, 1995; Coimbra et al., 1996; Colbourne et al., 1999c; Hickey et al., 2000; Corbett et al., 2002). Given these findings and the possibility of drug interactions it is remarkable that most studies did not assess post surgical temperature at all, including those using drugs known to affect temperature. Furthermore, of those that did, many only took rectal probe measurements for a short period following ischemia, or sampled temperature too infrequently (e.g., one sample at 24 hr after MCAO) or not long enough following drug administration (e.g., 15 min) to rule out temperature confounds. For instance, MK-801 can causes large fluctuations in temperature that oscillate between moderate hypothermia and normothermia (Colbourne et al., 1996). Such large temperature changes are easily missed with intermittent sampling. Furthermore, rectal temperature measurements can cause stress-induced fever, which occurs to differing levels in normal and ischemic animals (Clark et al., 2003). Thus, it is quite possible, and highly probable, that undetermined temperature alterations have confounded many cytoprotectant studies.

Several investigators placed animals in temperature-controlled chambers or rooms following ischemia, but surprisingly some did not then assess the animals' temperature. While this procedure would reduce the magnitude of hypothermia it would not prevent group differences nor prevent hyperthermia. Others claimed that the cytoprotective drug had been previously shown to have no temperature effect despite the fact that the temperature effects of a drug depend upon too many factors (e.g., dosing regimen,

ischemia injury, species, sex, etc.) to justify this claim. We strongly recommend that: 1) the method and exact duration of temperature measurement be clearly stated, 2) investigators should not rely upon historical data with respect to temperature effects of a drug, 3) investigators should not rely solely upon temperature data from a limited subset of animals, and 4) investigators measure temperature with telemetry probes that allow for repeated and non-stressful data collection. The latter allows for temperature confounds to be clearly ruled out, and is also necessary for precise control of temperature, which then lessens variability in outcome (Colbourne et al., 1999c).

There are several limitations of this survey that must be considered. First, we did not examine articles published in other journals. Given the high status (e.g., impact factor) of the journals surveyed we do not expect any different conclusions if other journals had been included in our survey and did not notice any differences among the journals that we did survey. Second, we cannot exclude the possibility that each treatment has been or would be investigated in a more thorough manner prior to clinical investigation. Our results simply indicate that this is not common. Third, we did not identify those differences between positive and negative findings for each drug that might account for the latter (e.g., absence vs. presence of temperature control). There were simply too few negative studies to do this. Fourth, we did not survey many other important factors that affect ischemic injury such as cerebral blood flow, plasma or CSF drug levels, glucose levels, etc. These must also be carefully considered in evaluating cytoprotection literature, but were beyond the scope of this survey. Finally, it could be argued that insufficient time has elapsed since the identification of these limitations (e.g., brief survival times) for experimental design to improve in the studies we examined. For

instance, the STAIR report (STAIR, 1999) was published in 1999 and thus investigators that adhered to these recommendations could only start publishing improved studies in late 2000 or 2001. We did not notice any trends within the 2000 – 2002 years and thus we grouped the data. Furthermore, given that the STAIR report was based upon many previous experimental findings and reviews, some published in the 1980's and others in the early 1990's, we feel that there has been more than sufficient time to allow investigators to improve their study design. It appears that neither those original reports nor STAIR (and perhaps this survey) has had a sufficient impact.

Experimental cytoprotectants have not translated well from the bench to the bedside. This translation failure is due to limitations and design flaws in both pre-clinical and clinical studies. While these limitations are widely recognized, the present survey, which characterized only a few of them, shows that many investigators have not taken these concerns seriously enough to better design rodent studies to avoid major physiological confounds and to better represent clinical conditions of cerebral ischemia. Notably, many studies failed to use: female or aged animals, behavioural assessment, longer survival times, or proper temperature control. Perhaps this is due to the increased costs and time associated with the use of longer survival times, behavioural assessments and physiological controls. As well, investigators feel the urge to publish findings quickly or may assume that other investigations will eventually examine these issues (e.g., whether protection is permanent).

Without significant changes to the design of rodent studies we expect further clinical failures. Thus, we strongly recommend that investigators more seriously consider these issues and that journal editors and reviewers at least insist upon better disclosure

and discussion of these methodological concerns. Finally, while there are no perfect experimental (or clinical) studies, we expect that every attempt to account for the major limitations and confounding factors discussed herein will result in better predictive accuracy and eventual clinical success.

Table 2.1

Study characteristics for recent (2000 – 2002) global and focal ischemia efficacy experiments. While many papers did not state the age, a weight range was commonly given or they stated "adult." Many of the ischemia studies that included females studied estrogen or related compound. No study examined sex differences in the efficacy of any other cytoprotectant. The data from 1990 are given in parenthesis within the Table.

	Global Ischemia	Focal Ischemia
Model Type	Rat 2-VO: 15% (37%) Rat 4-VO: 20% (21%) Gerbil BCCAO: 50% (42%) Mouse BCCAO: 15% (0%)	Intraluminal suture: • Rat: 50% (16.6%) • Mouse: 20.3% (0%) Clip occlusion: • Rat: 6.8% (0%) • Mouse: 0.8% (0%) Clot occlusion: • Rat: 11.0% (0%) • Mouse: 2.5% (0%) Electrocautery: • Rat: 5.1% (83.3%) • Mouse: 3.4% (0%)
Sex:	Male only: 75% (79%) Female only: 10% (5%) Both sexes: 5% (11%) Unstated: 10% (5%)	Male only: 83.1% (100%) Female only: 6.8% (0%) Both sexes: 3.4% (0%) Unstated: 6.8% (0%)
Age:	Stated: 10% (31.6%) Unstated: 90% (68.4%)	Stated: 20.3% (33.3%) Unstated: 79.7% (66.7%)

Figure 2.1

Percentage of 1990 and 2000 – 2002 studies categorized according to the longest survival time used and according to use of either a global (top) or focal (bottom) ischemia model. Of 20 global ischemia efficacy studies (2000 – 2002) only one was a negative finding at a 7-day survival time; thus that survival was sufficient. Another study found protection at a 7 but not a 28-day survival time.

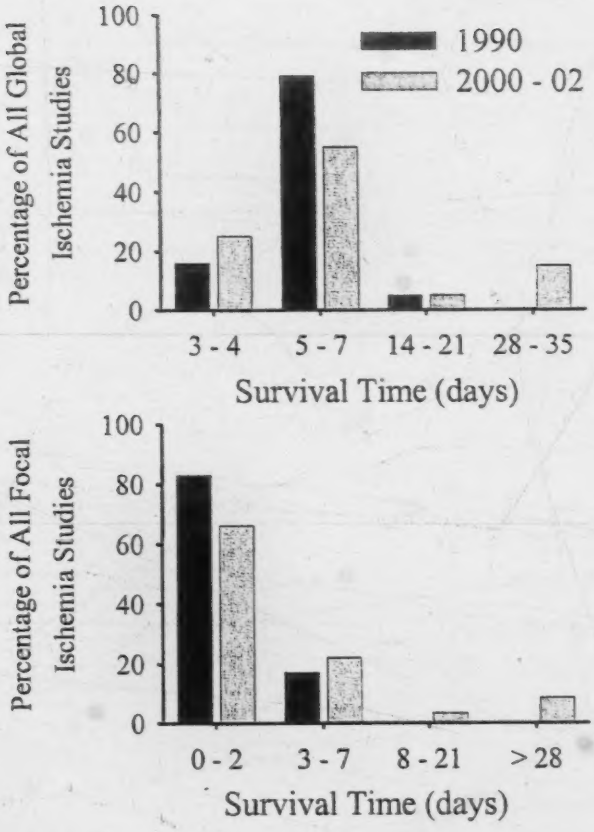
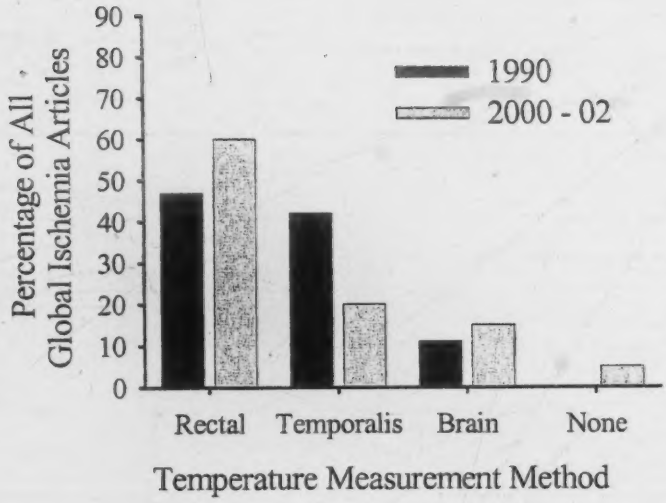

Percentage of All Global Ischemia Studies
100
80
60
40
20
0
1990
2000 - 02
3 - 4
5 - 7
14 - 21
28 - 35
Survival Time (days)
Percentage of All Focal Ischemia Studies
100
80
60
40
20
0
0 - 2
3 - 7
8 - 21
> 28
Survival Time (days)

Figure 2.2

Intra-ischemic temperature measurement methodology for global and focal ischemia models categorized as: 1) rectal or core temperature, 2) tympanic or temporalis muscle temperature (with or without rectal / core measurements), 3) direct brain temperature measurement (with or without other measures) or 4) unstated / none for 1990 and 2000 – 2002 inclusive.


Percentage of All Global Ischemia Articles
90
80
70
60
50
40
30
20
10
0
1990
2000 - 02
Rectal
Temporalis
Brain
None
Temperature Measurement Method

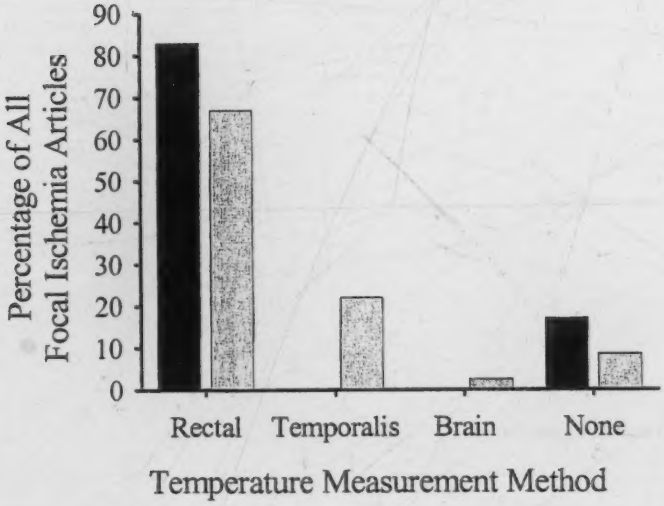
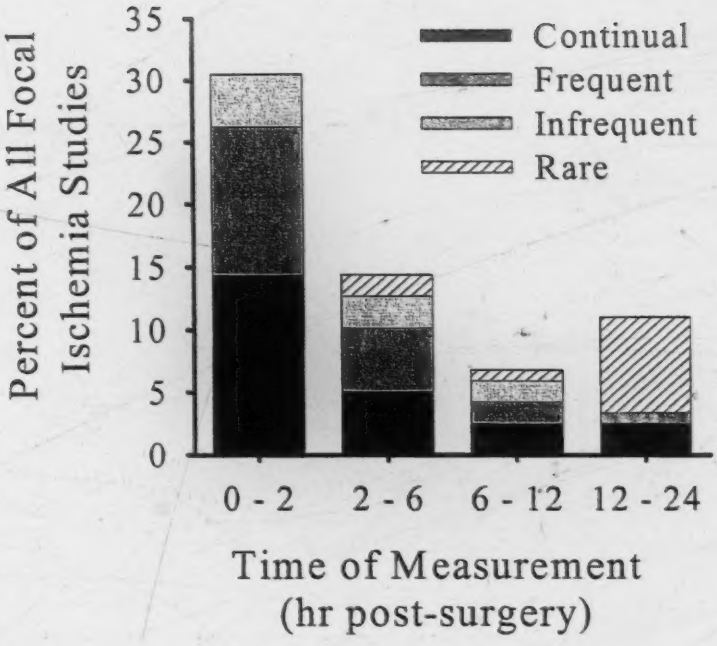

Percentage of All Focal Ischemia Articles
90
80
70
60
50
40
30
20
10
0
Rectal
Temporalis
Brain
None
Temperature Measurement Method

Figure 2.3

Percentage of focal ischemia cytoprotection studies (2000 – 2002) that measured temperature following surgery. None of the 1990 focal ischemia studies measured postoperative temperature. Data are broken down according to the time of the postoperative temperature measurement and by whether the sampling regimen was continual, frequent, infrequent or rare (see Methods). The reperfusion time was considered the "end of surgery" in those few studies that used prolonged anesthesia.


Percent of All Focal Ischemia Studies
35
30
25
20
15
10
5
0
Continual
Frequent
Infrequent
Rare
0 - 2
2 - 6
6 - 12
12 - 24
Time of Measurement (hr post-surgery)

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Chapter 3

Brain temperature measurement and regulation in awake and freely moving rodents

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Temperature significantly influences outcome from brain injury, especially ischemic insults. Although intraischemic hypothermia, of even a 1°C reduction, reduces brain injury (Busto et al., 1987b; Colbourne et al., 1997), hyperthermia is detrimental (Ginsberg and Busto, 1998). Furthermore, both delayed hypothermia and hyperthermia can significantly affect outcome in animal models of ischemia (for reviews see Colbourne et al., 1997; Ginsberg and Busto, 1998). Intra- and postischemic temperature changes often occur unintentionally as a result of putative neuroprotective drugs (e.g., MK-801; Buchan and Pulsinelli, 1990; Corbett et al., 1990; Colbourne et al., 1996), the anesthetic used (Colbourne et al., 1993c), the ischemia model (Colbourne and Corbett, 1994), and perhaps the method of measuring temperature. These unintentional temperature alterations are the most troublesome since they often go unnoticed. An additional confound is the sometimes discordant relationship between core and brain temperature measurements (Fig. 3.1) in rodent models (Busto et al., 1987a; Busto et al., 1989; Colbourne et al., 1993b), which also occurs in humans (Marion et al., 1997; Rumana et al., 1998). Despite clear data that rectal temperature does not always accurately predict brain temperature, many continue to rely solely on rectal temperature measurements during and after ischemia, or fail to measure temperature at all.

The surgical procedures (e.g., anesthesia) involved in cerebral ischemia models frequently introduce temperature artifacts. Additionally, ischemia itself affects postischemic temperature regulation, which then influences the extent and severity of brain injury and functional deficits. For example, forebrain global ischemia in gerbils results in a prolonged period (12 – 24 hr) of hyperthermia, the duration of which depends upon the severity of ischemia (Colbourne and Corbett, 1994; Colbourne and Corbett,

1995). Conversely, global ischemia in the rat, via the 4-vessel occlusion (4-VO) method, can result in a prolonged period of mild hypothermia (Colbourne et al., 1999b). Similar results have been observed in a cardiac arrest model in rat (Hickey et al., 2000). These studies used radiotelemetry (AM signals emitted from an implanted device) core or brain temperature probes, which provides for reliable and less stressful temperature measurement. The two-vessel occlusion model (2-VO) of global ischemia in rat is thought to produce delayed hyperthermia (Coimbra et al., 1996), but these findings have not been confirmed with telemetry temperature probes. Given that rectal temperature probes cause a rise in body temperature (stress-induced fever; Cabanac and Briese, 1992) such findings in the 2-VO should be confirmed with radiotelemetry.

Focal ischemia in rat is also thought to cause hyperthermia (Zhao et al., 1994; Reglodi et al., 2000) aggravating injury. This has not been consistently found, however, with rectal temperature measurements (Schmid-Elsaesser et al.). Furthermore, recent studies with distal middle cerebral artery occlusion (MCAO) via clip occlusion (Colbourne et al., 2000) and intraluminal MCAO (Corbett et al., 2000; Oryu, Colbourne, and Auer, unpublished data) did not substantiate the occurrence of any postischemic hyperthermia. Such differences are likely due to model differences (e.g., involvement of hypothalamus), but may also, in part, be related to the method of measuring temperature. For example, rectal probe insertion, which causes temperature to rise (Cabanac and Briese, 1992), may affect ischemic and normal animals differently. In gerbils subjected to unilateral common carotid artery occlusion (Colbourne and Auer, unpublished data), those that suffered a focal ischemic insult experienced a variable postoperative temperature profile with sometimes-marked fluctuations between normothermia and

hypothermia (e.g., 32°C). Telemetry data in mice stroke models are needed.

Given the above findings, it is clear that most ischemia studies do not adequately measure or control temperature. This article discusses our experience with the use of telemetry brain and core temperature probes for temperature measurement, and a new method to effectively regulate temperature during and after cerebral ischemia in the conscious animal. Some advantages of the telemetry system include: ability to measure temperature without causing stress to the animal, continual data sampling and storage, rapid response to temperature changes and the ability to use an automated feedback system for temperature control. Major disadvantages are the cost, and the need for additional surgery.

Telemetry Probe Models

Several models and manufacturers of core temperature probes exist for gerbil or mouse (e.g., Model TA10TA-F20; Data Sciences Int., St. Paul, MN, USA), and for rat (e.g., Model TA10TA-F40; Fig. 3.2). Similarly, brain temperature probes for gerbil or mouse (Model XM-FH; Mini Mitter Co., Inc., Bend, OR, USA), and for rat (Model; VM-FH; Fig. 3.2) are also available. While all probes are calibrated prior to shipping, calibration (in a water bath) against a laboratory standard is recommended. In our laboratory, calibrations are done using a thermocouple probe (Model HYP1-30-1/2-T-G-60-SMP-M) and thermometer (Model HH-21 Microprocessor Thermometer; Omega Engineering, Stamford, CT, USA). Probes must also be recalibrated after a battery change.

Surgical Procedures

Core Probe Implantation

Chemically sterilized (with 2% glutaraldehyde solution soak for > 12 hr) core probes are easily and quickly implanted under inhalation anesthetic via a small abdominal incision. Following surgery, animals are allowed to recover for at least 4 days before further experimentation to allow circadian rhythms to normalize. In our experience, core probe implantation does not affect the average daily temperature of the animal, but may flatten the fluctuations in temperature over the course of the day/night cycle for approximately 1 to 2 days. Furthermore, this procedure often causes a small drop in body weight, which recovers over a few days.

Brain probe implantation

In gerbils and mice, brain probes can be glued directly to the skull or inserted through a previously affixed cannula (Fig. 3.3). Typically, in the latter method, three nylon screws (MN-080-2; Small Parts Inc, Miami Lakes, FL, USA) are glued (cyanoacrylate glue) to the skull and then dental cement is used to secure the cannula (a 20-gauge needle cut to desired length) to the screws (see Colbourne et al., 1996). The brain probe is secured to the cannula with a loop of tape. Unfortunately, with this method, dental caps may become dislodged after 4 – 7 days due to the stress on the glued contact points (e.g., when a gerbil sleeps and is in a curled position). Additionally, the shaft of brain probes may bend or break. Thus, more recently, we have glued the brain probe to a plastic nut, which is then screwed into a cannula (C311G 20G; Plastics One Inc., Roanake, VA, USA; Figure 3.3A). The cannula can be secured as previously described, or with small nylon screws (Small Parts Inc.) threaded into the skull, affixed with cyanoacrylic glue, and secured with dental cement. Care must be taken to ensure the screws are cut to just the thickness of the skull to avoid injury to the brain. Having the

screws secured to the skull provides a more durable headcap and is less likely to result in brain probe damage. Also, this new method allows for insertion and removal of the probe (under brief anesthesia) at any time, thus decreasing the risk of breakage and allowing it to be used in other animals.

In rats, a guide cannula similar to the one used in gerbil and mouse is secured to three metal screws (Model MX-080-2; Small Parts) with dental cement. This procedure however, requires a plastic cylinder (e.g., 10-mm length of a 5-cc syringe) secured with dental cement around the probe to prevent the rat from breaking the probe. Since the probe is secure within the syringe, the nut (as described for mouse or gerbil) is not used. If needed, a rubber plunger from the syringe is placed above the probe keeping it in place (Colbourne et al., 1996).

Somnotol (sodium pentobarbital; 65 mg/kg) is the most commonly used anesthetic for these procedures since it keeps the animal immobile for hours, allowing the dental cement to harden. If inhalation anesthetic is used, the animal must be administered a pain reliever, as recovery is much quicker than with Somnotol. Not surprisingly, however, Somnotol causes significant hypothermia that may need to be monitored and avoided. Inhalation anesthetics such as halothane and isofluorane will also cause hypothermia.

Temperature Measurement and Control

Different techniques have been used to maintain temperature during ischemia. Most commonly, core body temperature is maintained using a rectal probe and heating lamp or electric blanket. As discussed, core and brain temperatures can dissociate especially during global ischemia (Busto et al., 1987a; Colbourne et al., 1993a;

Colbourne et al., 1993b). Therefore, brain temperature is regulated during surgery with an automated system originally described in Colbourne et al. (Colbourne et al., 1996). Briefly, temperature signals from the telemetry probe are sampled every 30 s, ensuring strict control of temperature. Less frequent sampling will result in more variable temperature control. An infrared lamp or heated water blanket is then activated when temperature falls below a specified value (e.g., 36.5°C). If a lamp is used, care should be taken that it is placed directly above the animal at a sufficient distance to avoid burning the animal. Heated water blankets (Gaymar Industries Inc, Orchard Park, NY, USA) can also be used effectively (Colbourne et al., 1993b). Electric blankets are not recommended as they often interfere with the telemetry probe signal.

Temperature regulation following ischemia is also required and necessitates the use of a telemetry system. Precise temperature control in conscious rodents is only possible if temperature is frequently measured. This is not practical with rectal probe measurements. Precise temperature regulation is achieved with the automated telemetry system that uses fine water mist and overhead fans (Model TNE2A) to cool, and infrared lamps (175 – 250 W) to heat (Fig. 3.4). The duration of fan and lamp usage is set to 30 s (i.e., entire intersample cycle), whereas the amount of water spray is varied according to several parameters (e.g., difference between current and desired temperatures) as discussed previously (Colbourne et al., 1996). Each device (e.g., lamp) is separately controlled by custom software that obtains the temperature data from the DataSciences ART program's temperature data storage files (see DSI technical information for more information on data storage format or contact F.C.). This software then controls the lamp, fan and water spray (solenoid-controlled valves; valves are of the two way normally

closed type) devices through a KPC-PIO-24 card (Keithley Metrabyte, Taunton, MA, USA) that controls ac relays (Model SMOAC5) mounted on an SSIO-24 board (or similar setup). Each relay independently controls a device. Readers are referred to Colbourne et al., 1996 for further details on system setup (e.g., location of fans, possible problems with regulation).

Application of Radiotelemetry in Ischemia

Measurement and control of brain temperature during ischemia are essential as emphasized by many authors (e.g., Busto et al., 1987a). Such control is easily achieved with inexpensive equipment (e.g., thermocouple probe and heating lamp). However, the regulation of postischemic temperature, which is often just as necessary, is more difficult and more expensive. Herein, we described our use of telemetry temperature probes and an automated system that used lamps, fans and fine water mist to regulate temperature in rodents. Telemetry probes allow for repeated and accurate measurements of temperature not easily achieved with other devices (e.g., standard thermocouple probes). For instance, rectal probe measurements, while giving a valid (within 0.3°C) measure of core temperature, result in a stress-induced fever, which may aggravate injury and confound the study. Additionally, rectal probe temperature measurements are not practical for continual measurement, which is essential for accurate temperature control. Thus, we strongly urge researchers to use telemetry temperature probes and to regulate temperature in an effort to ensure model consistency (Colbourne et al., 1999b) and to avoid confounding results (e.g., with drugs or procedures that alter temperature); both are problems that have plagued the ischemia literature.

The choice of using either core or brain temperature probes is not always easy.

Given the potentially large dissociations between core and brain temperature during *global* ischemic insults (e.g., Busto et al., 1987a; Colbourne et al., 1993c) we recommend that brain temperature be either measured directly (e.g., with a wired thermocouple probe or telemetry probe) or with a validated surrogate measure (e.g., tympanic membrane temperature). Postischemic brain temperature can be either directly measured or estimated with core telemetry probes (Fig. 3.2). The average baseline temperature of normal rats and gerbils are similar, but the average core temperature is typically 0.5 – 1°C higher than average brain temperature. This concurs with measurements of brain and core temperature taken nearly simultaneously (within 30 s) with dually implanted core and brain temperature telemetry probes in rats (Fig. 3.1). In this case core temperature was about 0.7°C higher than brain temperature with a high correlation ($r = 0.97$) between the two measures. However, preliminary data suggest that the relationship between core and brain temperature is somewhat less consistent when rats are subjected to externally imposed hypothermia (Colbourne, unpublished data).

Brain temperature measurement is also possible during and after focal ischemia in many animal models including gerbils, mice, and rats. The use of brain probes is more difficult in the clip-occlusion MCAO model in rat due to the need to estimate blood flow near the brain probe assembly. Brain probes can easily be used in the intraluminal suture occlusion MCAO model, a method usually applied in rats (Corbett et al., 2000). The mouse is the species used in knockout gene experiments. Brain probes can also be used in mice, but small size and weight make this more challenging in mouse than in a gerbil or rat. There is a paucity of data on the relationship between core and brain temperature in mouse and rat MCAO models. Similarly, there is no consensus on which region to

measure within the brain, during or after focal ischemia. We suspect that there may be biologically significant temperature dissociations between ischemic areas (e.g., the core) and normal, contralateral regions.

Temperature measurement and control during ischemia is obviously important. However, it is not clear for how long postischemic temperature should be monitored or controlled. We routinely measure temperature for 4 – 7 days following ischemia. Given that delayed hypothermia (e.g., by 12 h after global ischemia; Colbourne et al., 1999a) is cytoprotective and delayed fever is detrimental (Ginsberg and Busto, 1998), temperature measurement for at least 2 postoperative days is strongly recommended. However, this may need to be extended after insults that result in a slower maturation of damage. We further recommend that temperature be measured at least every 30 min, or more frequently if temperature changes are being imposed (e.g., every 30 s if hypothermia is being induced with the methodology described herein). Rapid fluctuations may occur because of the ischemia model or because of a drug therapy. For example, MK-801 induced rapid and substantial temperature alterations in gerbils that could easily be missed if temperature were sampled only every couple of hours (Colbourne et al., 1996).

Investigations into the efficacy and mechanisms of postischemic hypothermia have used a number of methods to regulate temperature (e.g., cold room, drugs). In our opinion, the use of a rapid feedback system with lamps, fans, and water spray results in a more precise temperature control than other methods. Typically, our method is able to maintain temperature within ±0.5°C of the desired value. This is impossible to achieve with pharmacological agents or by placing animals in cold rooms. Whereas an anesthetic (e.g., isoflourane) aids the production of brief hypothermia (e.g., < 6 hr), in rodents it is

not possible to maintain animals under anesthesia for periods long enough to study which durations of hypothermia are most effective (e.g., 2 days of mild hypothermia). Our system also allows multiple animals to be simultaneously regulated, which would be difficult if an anesthetic is used. Regardless, further study is needed on pharmacological methods of inducing hypothermia, with and without external regulation, as these will be preferred in the clinic and, it is hoped, will be more efficacious and less stressful.

In summary, prolonged temperature measurement and regulation are essential in ischemia studies. Given the positive findings of prolonged hypothermia after cardiac arrest (Bernard et al., 2002; The Hypothermia After Cardiac Arrest Study Group, 2002) and stroke (Schwab et al., 1998) it is likely that more investigators will need accurate methods to control temperature.

Figure 3.1

Temperature measurements from both core and brain (cortex) telemetry probes in the same rat. Readings were taken approximately every 5 min at three different times of day (morning: 6 - 7 AM, mid-day: 12 – 1 PM, evening: 6 – 7 PM). Brain temperature was recorded and followed by core temperature sampling ~ 30 s later.

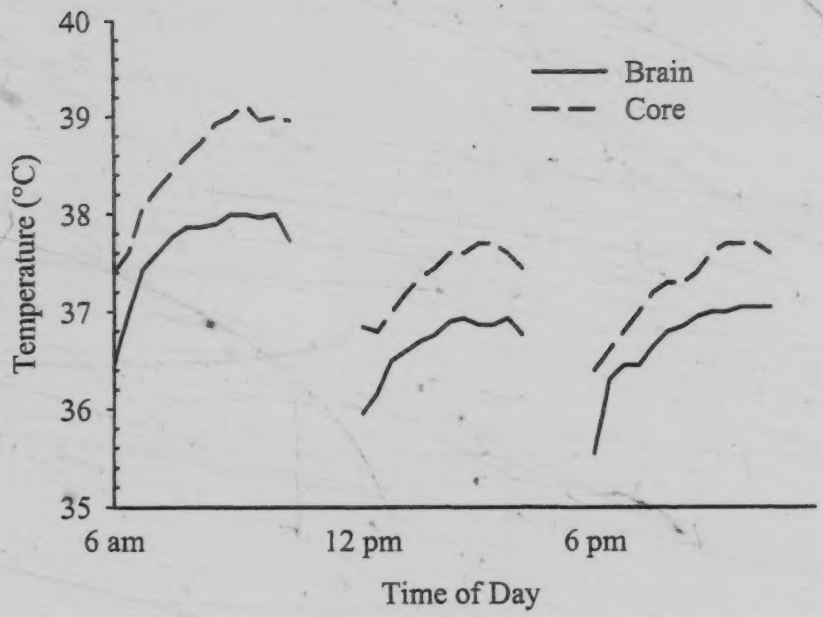

40
39
38
37
36
35
Temperature (°C)
Brain
Core
6 am
12 pm
6 pm
Time of Day

Figure 3.2

Brain telemetry probes (Mini-Mitter Co. Inc, USA) and core telemetry probes (Data Sciences International Inc., USA). From left to right: brain probe Model XM-FH for gerbil or mouse; brain probe Model VM-FH for rat; core probe Model TA10TA–F20 for gerbil or mouse; core probe Model TA10TA–F40 for rat. Black bar = 3 cm.

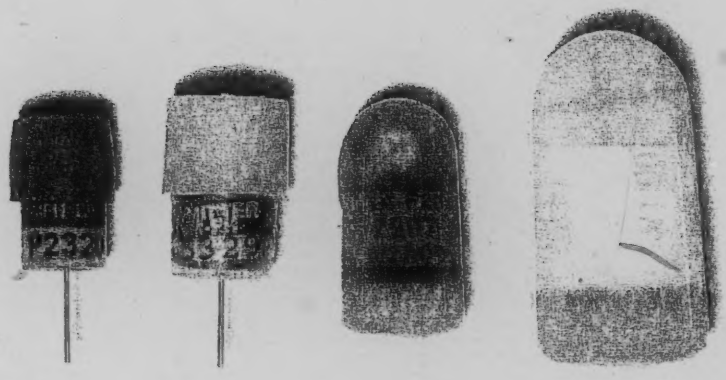
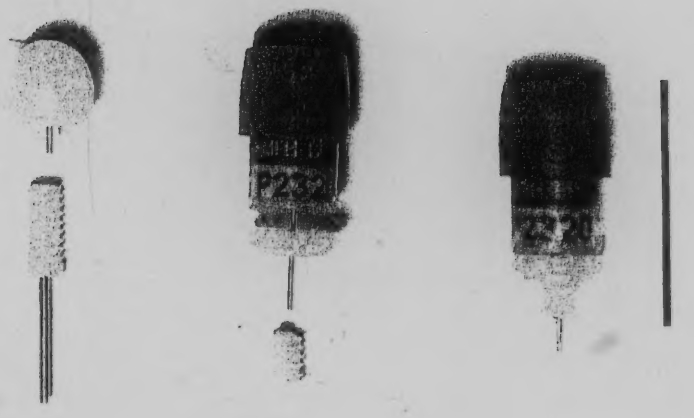


Figure 3.3

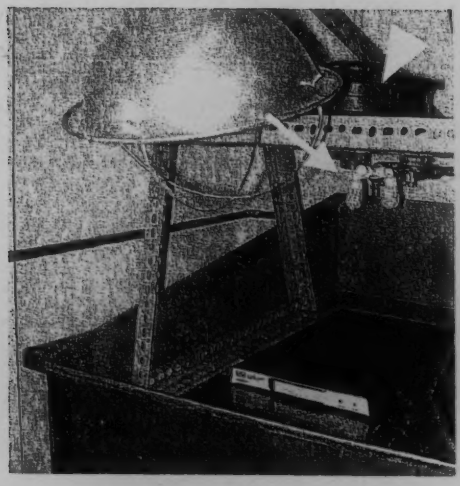
A. Cannula (20 gauge Model C311G; Plastics One, USA) with stylet (left) and brain probe with nut affixed (right). **B.** Gerbil/mouse brain probe attached to cannula (cut according to size), as it would be seen in the animal. Black bar = 3 cm.



A B

Figure 3.4

Photograph of temperature system, including receiver, overhead spray mister (white arrowhead), fan (white arrow) and infrared lamp.



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Chapter 4

Stress Induced Fever after Postischemic Rectal Temperature Measurements in the Gerbil

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Global (e.g., cardiac arrest) and focal (e.g., embolic stroke) cerebral ischemia is a leading cause of morbidity and death. Of many factors affecting outcome, temperature is one of the most important determinants (Colbourne et al., 1997; Ginsberg and Busto, 1998). Intra- and postischemic hypothermia improves outcome whereas hyperthermia worsens it. Accordingly, intra- and postoperative rectal temperature (T_{rec}) measurements are commonly used in rodent ischemia studies (DeBow et al., 2003).

Core temperature (T_{core}) should not be used to estimate brain temperature (T_{brain}) during anesthesia and global ischemia since they can dissociate by 5 – 6°C (Busto et al., 1987). In non-anesthetized rodents T_{core} accurately reflects T_{brain} (Dilsaver et al., 1992; DeBow and Colbourne, 2003). However, obtaining T_{rec}, which estimates T_{core} in conscious rodents, causes a stress-induced fever (SIF), which is due to an elevated temperature set-point resulting in a controlled rise in temperature (Kluger et al., 1987; Briese and Cabanac, 1991; Cabanac and Briese, 1992). This SIF has not been examined in the gerbil, which is commonly used to model global ischemia (DeBow et al., 2003). Furthermore, the pattern of SIF in ischemic versus non-ischemic gerbils is unknown. We hypothesized that T_{rec} measurements would cause a SIF in all gerbils, but that the SIF would be attenuated in ischemic gerbils due to the presence of spontaneous postischemic hyperthermia (Colbourne and Corbett, 1994). The SIF resulting from T_{rec} measurements may worsen histological outcome as fever aggravates injury (Ginsberg and Busto, 1998).

Materials and Methods

Subjects

Forty-five female Mongolian gerbils (60 – 90 g, Charles River, St. Constant, QUE, Canada) were used. All procedures were in accordance with the Canadian Council for Animal Care Guidelines. Four of these gerbils were excluded due to surgical error.

Implantation Procedure

Animals were anaesthetized with sodium pentobarbital (65 mg/kg i.p.) for implantation of a telemetry probe (Model TA10TA-F20, Data Sciences Int., USA) into the abdominal cavity. Additionally, a 5-mm guide cannula was fixed to the surface of the skull at one mm anterior and one mm lateral to bregma as previously described (DeBow and Colbourne, 2003).

Ischemia and Temperature Measurement

Between 3 – 7 days following implant surgery, gerbils were anesthetized with isoflorane (~1.5% maintenance in 70% N_2O and 30% O_2) and randomly assigned to receive ischemia (5-min bilateral common carotid artery occlusion, ISC) or sham surgery (carotid isolation only, SHAM) as previously described (Colbourne and Corbett, 1994). Brain temperature was maintained at ~36.0°C (via overhead infrared lamp) using a thermocouple needle probe (model HYP1-30-1/2-T-G-60-SMP-M, Omega Engineering, CT, USA) inserted into the dorsal lateral striatum (via previously implanted cannula).

A lubricated thermocouple probe (model RET-3, Physitemp Instruments, Inc., NJ, USA.) was used to measure T_{rec} at 1, 2, 3 and 4 hours post-ischemia (ISC-T_{rec}) or sham occlusion (SHAM-T_{rec}), and at the same times on the following three days (i.e., 16 samples total over 4 days; ~30 sec procedure). The T_{rec} samples were obtained in gently restrained, conscious gerbils. Control sham (SHAM-C) and ischemic (ISC-C) animals did not have T_{rec} measured. The T_{core} was sampled and recorded every 30 seconds (2 second

averages) with an ART data acquisition system (Data Sciences, Int.) for several days prior to and 96 hours following surgery in all animals. The peak T_{core} change (SIF) in SHAM-T_{rec} and ISC-T_{rec} groups was determined by averaging the 15 – 30 minutes following the T_{rec} measurement, corresponding to the maximal temperature rise, and subtracting from this value the average temperature for the same period in the respective control group. The peak change in T_{core} following the four T_{rec} measurements was averaged for each day since there were no notable differences with each day (i.e., no time effect within a day; repeated measures ANOVA). Accordingly, an ANOVA on the peak SIF was done on the T_{rec} groups over four days (mixed ANOVA).

Histology

Seven days after ischemia / sham occlusion gerbils received an overdose of sodium pentobarbital (~ 80 mg/kg i.p.) and were transcardially perfused with 10% formalin. Brains were frozen, sectioned coronally (10 μm), and stained with Cresyl violet. CA1 sector cells were counted and summated from the medial (adjacent to subiculum), middle (apex) and lateral (next to CA2) sections of CA1 at -1.7 mm to bregma. Data were analyzed with ANOVA with two factors (SHAM vs. ISC; T_{rec} measurements vs. none).

Results

The average baseline T_{core} (24 hr average) for all animals was 37.5°C ± 0.2 (SD) and there were no significant main effects or interaction (p 0.498). The T_{brain} during surgery, which was regulated, was also not significantly different (p 0.073) among groups, with an overall average temperature of 36.6°C ± 0.09. As expected, the ISC-T_{rec} (38.3°C ± 0.4; average temperature during the first 6 hr after ischemia) and ISC-C

(38.2°C ± 0.6) groups experienced significant ($p = 0.004$) spontaneous postischemic hyperthermia (T_{core}) compared to SHAM- T_{rec} (37.9°C ± 0.5) and SHAM-C groups (37.6°C ± 0.5). The T_{core} was similar among groups on subsequent days (data not shown).

The T_{rec} measurements caused a SIF (T_{core}) that was greater in the SHAM-T_{rec} than the ISC-T_{rec} group ($p = 0.030$; Figure 4.1). There was a significant Day main effect ($p < 0.001$) indicating that the SIF differed among the four days. For example, a simple effects analysis showed that the SIF on day 1 was less than on other days ($p < 0.001$). This effect appears to be largely due to the ISC-T_{rec} group that had an increasing SIF over days (Figure 4.1) although the Day by Group interaction only approached significance ($p = 0.081$). At its maximum the SIF response peaked at approximately 1°C with a length of about 1 hour. The SIF response did not change over the four samples taken within each day.

A two-way ANOVA on hippocampal CA1 cell counts revealed a significant main effect for insult (SHAM vs. ISC; $p < 0.001$), but no significant T_{rec} measurement effect ($p = 0.349$) or interaction ($p = 0.905$). Thus, the ISC-T_{rec} (28.2% of normal CA1 cells remaining) and ISC-C (36.8%) groups were not significantly different.

The difference between T_{core} and T_{rec} measurements (i.e., $T_{core} - T_{rec}$) were 0.2°C ± 0.3 and 0.2°C ± 0.3 in SHAM-T_{rec} and ISC-T_{rec} groups, respectively ($p = 0.709$). Additionally, the T_{rec} measurements correlated well with the T_{core} (Figure 4.2) taken immediately before the rectal probe insertion in the SHAM-T_{rec} ($R = 0.904$, $p < 0.001$) and ISC-T_{rec} ($R = 0.880$, $p < 0.001$) groups. The slopes and intercepts of the regression lines did not differ significantly between these groups ($p = 0.155$).

Discussion

The primary finding of this study is that postischemic T_{rec} measurements caused a different SIF profile in SHAM-T_{rec} and ISC-T_{rec} gerbils. The SHAM-T_{rec} gerbils experienced a SIF with a peak of approximately 1°C lasting about one hour; a profile that did not habituate even after 16 T_{rec} measurements. The SIF was initially prevented in ISC-T_{rec} gerbils, but eventually returned to SHAM-T_{rec} levels. Finally, T_{rec} measurements accurately reflected the concurrent T_{core} in SHAM-T_{rec} and ISC-T_{rec} groups.

Gerbils frequently experience spontaneous postischemic hyperthermia after forebrain ischemia (Colbourne and Corbett, 1994; present data). Perhaps this hyperthermic response resulted in a ceiling effect (Feng et al., 1989) whereby further rises in T_{core} due to T_{rec} measurements were blocked. This, however, cannot explain the attenuated SIF on day 2 since ISC-T_{rec} gerbils were then normothermic. More likely, ISC may have transiently affected molecular mechanisms of antipyresis, such as glucocorticoid release (Kozak et al., 2000), or fever generation. Further studies are needed to determine the mechanisms of spontaneous postischemic hyperthermia and the postischemic SIF response.

The occurrence of a SIF after T_{rec} measurements questions the utility of this method in brain injury studies, especially ischemia research. Delayed hyperthermia aggravates ischemic injury (Ginsberg and Busto, 1998) and the occurrence of multiple SIF responses due to T_{rec} measurements may under certain circumstances aggravate brain damage. Presently, T_{rec}-induced SIF did not occur until the second day, which likely accounts for the lack of effect on CA1 damage. However, T_{rec} measurements at other times (e.g., 12 – 24 hr) may aggravate injury in this model. Furthermore, since the SIF

response likely varies with ischemia model and insult severity, the occurrence of spontaneous temperature changes (i.e., hypothermia or hyperthermia as a result of the insult), and the presence of drugs (e.g., antipyretics Briese and Cabanac, 1991), it is quite possible the unknown SIF response may have confounded many studies.

In summary, T_{rec} measurements accurately predict T_{core} in normal and ischemic gerbils. However, T_{rec}–induced SIF differed between SHAM-T_{rec} and ISC-T_{rec} groups and over test days. Studies that rely upon multiple T_{rec} measurements may be confounded. Therefore, our findings illustrate the need for non-stressful methods of measuring postoperative temperature, such as the use of telemetry probes, and perhaps the need to avoid early behavioural manipulations that can also cause a SIF (Colbourne et al., 1998).

Figure 4.1

Mean T_{core} (°C) for one hour (5 min averages) following the T_{rec} measurements on days 1 – 4 following ischemia / sham occlusion surgery. Data were averaged over the four T_{rec} measurements taken each day.

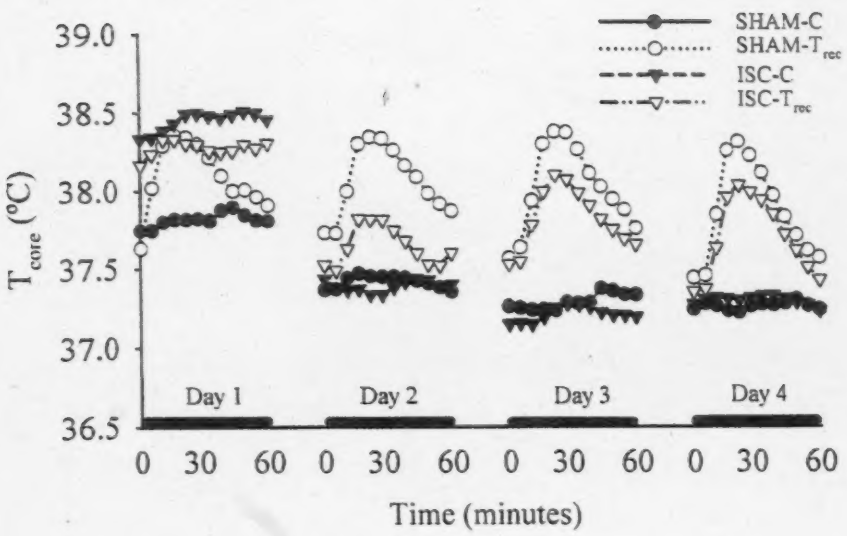
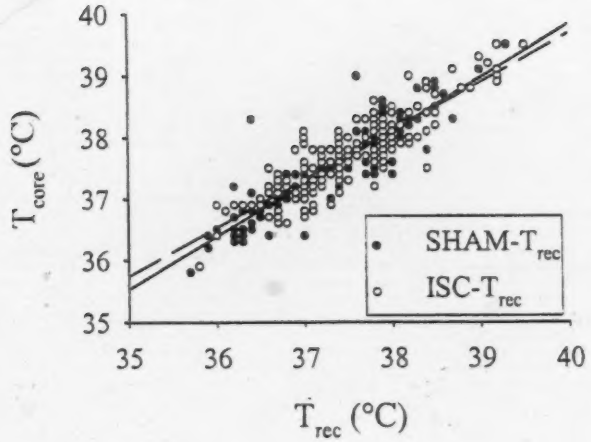
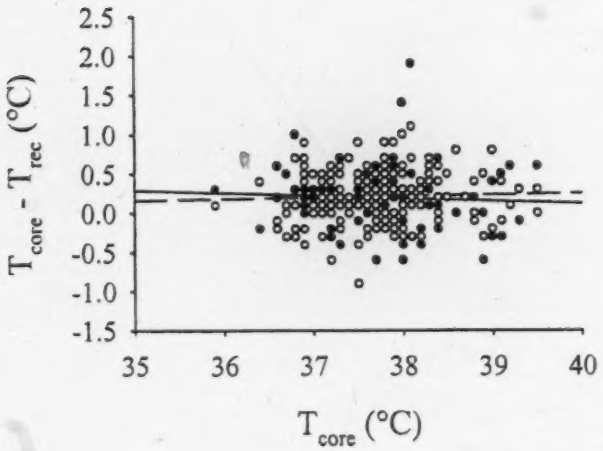

SHAM-C
SHAM-T$_{rec}$
ISC-C
ISC-T$_{rec}$
39.0
38.5
38.0
37.5
37.0
36.5
T$_{core}$ (°C)
Day 1
Day 2
Day 3
Day 4
0 30 60 0 30 60 0 30 60 0 30 60
Time (minutes)

Figure 4.2

Relationship between T_{rec} and T_{core} (°C) in SHAM-T_{rec} and ISC-T_{rec} groups (top). The T_{rec} was highly predictive of T_{core} in both SHAM-T_{rec} (dashed regression line) and ISC-T_{rec} (solid line) groups. There are 16 samples per animal (4 per day over 4 days). The bottom graph shows the agreement of T_{rec} and T_{core} measures (i.e., $T_{core} - T_{rec}$) over the range of T_{core} values. The T_{rec} value averaged 0.2°C lower than T_{core}.


40
39
38
37
36
35
T_{core} (°C)
SHAM-T_{rec}
ISC-T_{rec}
35
36
37
38
39
40
T_{rec} (°C)


2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
T_{core} - T_{rec} (°C)
35
36
37
38
39
40
T_{core} (°C)

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Chapter 5

Delayed transient ischemic attacks kill some CA1 neurons previously salvaged with postischemic hypothermia: neuroprotection undone.

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Forebrain ischemia results in delayed degeneration of hippocampal CA1 sector neurons in both humans (Petito et al., 1987) and rodents (Kirino, 1982; Pulsinelli et al., 1982). This delayed neuronal death is believed to be due, in part, to an increase in glutamate release during ischemia (Silver and Erecinska, 1992) activating both *N*-methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoazole-proprionic acid (AMPA) receptors. Notably, cell death is preceded by downregulation of the AMPA receptor subunit GluR2, making the channel permissive to Ca^{2+} and Zn^{2+} entry, and therefore continuing toxicity (Tanaka et al., 2000). Further support for this comes from studies that show that depolarization of postischemic CA1 neurons leads to cell death, which is related to abnormal regulation of Ca^{2+} (Andine et al., 1988, 1992; Silver and Erecinska, 1992; Tsubokawa et al., 1992).

Intra-ischemic hypothermia permanently reduces CA1 neuronal death and is unparalleled in efficacy (Dietrich et al., 1996). Brief postischemic hypothermia only transiently reduces cell death (Dietrich et al., 1993). Prolonged postischemic hypothermia (e.g. 24 - 48 h) can substantially attenuate CA1 neuronal death, even when delayed 12 h after ischemia (Colbourne and Corbett, 1994; Colbourne and Corbett, 1995; Colbourne et al., 1999; Hickey et al., 2000). However, some initially salvaged neurons eventually die (Colbourne and Corbett, 1995; Colbourne et al., 1999). Furthermore, some 'rescued' CA1 cells are morphologically abnormal (e.g., organelle dilations, mitochondrial injury; Colbourne et al., 1999). In addition, while prolonged hypothermia induced after global ischemia (1-h delay) attenuates GluR2 downregulation in gerbils and promotes an eventual full recovery, there was still a significant GluR2 downregulation that lasted for several days (Grooms et al., 2000). These, and likely other abnormalities in rescued CA1

neurons, may account for the very slow cell death in some neurons, possibly by repeated neuronal activation (e.g., spatial learning tasks). Furthermore, one would expect that such abnormal neurons might be especially sensitive to subsequent ischemic insults, including insults that would not normally kill CA1 neurons. This is supported by the fact that a single TIA (2 min) acts synergistically with GluR2 downregulation (via antisense oligodeoxynucleotide knockdown) to kill CA1 neurons (Oguro et al., 1999). Thus, hypothermia-rescued CA1 neurons, which have a transient GluR2 downregulation among other abnormalities, should be especially vulnerable to delayed transient ischemic attacks (TIAs) that would not normally kill CA1 cells. A loss of previously protected CA1 neurons has been demonstrated recently (Farrell et al., 2001). In that study, environmental enrichment delayed for 3 days following ischemia partially diminished neuroprotection achieved earlier with ischemic preconditioning.

In order to test the resiliency of hypothermia-treated CA1 neurons after ischemia, gerbils were subjected to delayed TIAs (1.5 min) on days 5 and 6 *after* they were subjected to a severe global ischemic insult (5 min) that was treated with prolonged postischemic hypothermia. A 12-h intervention delay was used in order to produce a partial sparing of CA1 neurons (Colbourne et al., 1999) and thus optimize the conditions for assessing delayed TIAs. Prolonged hypothermia induced soon after ischemia would be more resilient to exacerbation as most cells are rescued permanently (Colbourne et al., 1999). We chose two 1.5-min TIAs since they alone do not kill CA1 neurons in normal gerbils, but do sufficiently prime the cells to produce ischemic preconditioning (Corbett and Crooks, 1997). The TIAs were administered on days 5 and 6 because of several cellular abnormalities, discussed above, known to occur at this time and since the

hypothermia treatment extended over 3 days. The 24-h interval between TIAs was chosen to avoid the obviously deleterious effects of repeated, brief and untreated ischemic insults when they occur closely together (Lin et al., 1992). Thus, we sought to determine whether there is an enhanced and persistent vulnerability of *salvaged* CA1 neurons to otherwise *non-lethal* TIAs.

Materials and Methods

Subjects

A total of 76 (see Fig. 5.1 for groups and numbers) female Mongolian gerbils (64.8 g ± 6.0 g [mean ± S.D.]; Charles River, Quebec, Canada) were used in accordance with the Canadian Council for Animal Care guidelines and the Biosciences Animal Policy and Welfare Committee at the University of Alberta. Three gerbils were excluded due to surgical complications. All gerbils were on a 12-hour dark-light cycle with food and water ad libitum.

Ischemia and Temperature Control

Gerbils were first implanted with sterilized telemetry probes (model TA10TA-F20, Data Science, St Paul, MN, USA), which were placed into the peritoneal cavity under sodium pentobarbitol anesthesia (65 mg/kg i.p., and 0.05 mg/kg s.c. atropine sulphate). Additionally, a 20-gauge stainless steel guide cannula was implanted on the dural surface overlying the frontal cortex of the left hemisphere (~ 1 mm anterior and lateral to bregma). The cannula was placed such that an inserted 30-gauge thermocouple probe (Model HYP1-30-1/2-T-G-60-SMP-M; Omega Engineering, Stamford, CT, USA) sampled dorsal striatal temperature during ischemia and sham surgery. Upon removal of the thermocouple probe following surgery, a dummy cannula was inserted to prevent

infection. Following this surgery all gerbils were individually housed in cages and placed upon receivers (RPC-1; Data Sciences) which sampled core temperature every 30 s. The data collected the day immediately before ischemia and sham surgery served as baseline.

Brain temperature telemetry probes are susceptible to damage following prolonged use in a gerbil, and thus core telemetry probes were used in this study. During ischemia in gerbils, core temperature does not necessarily predict brain temperature (Colbourne et al., 1993), but the relationship is good in conscious postischemic rodents (DeBow and Colbourne, 2003). Brain temperature control during injury reduces variability in this model, and thus both brain and core temperature measurements were taken during the time of ischemia.

Four days following core probe and brain cannula implantation, gerbils were anesthetized with 4% (1.5 – 2% maintenance) isoflourane anesthetic in 70% N_2O and 30% O_2. Brain and core temperature were monitored and maintained with an infrared heat lamp near 36.5°C and 37.5°C, respectively. Striatal temperature was measured via the thermocouple probe, and core via the telemetry abdominal probe. Gerbils were randomly assigned to receive ischemia (5-min bilateral common carotid artery occlusion, ISC) or sham surgery (arteries were isolated but not occluded, SHAM). Both common carotid arteries were occluded in ISC gerbils using micro-arterial clamps. At the end of the occlusion, the clamps were removed, the arteries visually inspected for reflow, the midline incision sutured, the thermocouple probe removed and anesthesia was discontinued.

All gerbils received post-ischemic temperature control (Fig. 5.2), excluding untreated SHAM animals as they were allowed to self regulate (SHAM+NORMO). In an

effort to minimize variability, untreated ischemia groups (ISC+NORMO) were regulated for 12 h to mimic a mild hyperthermia pattern that spontaneously occurs (Colbourne and Corbett, 1994; Colbourne and Corbett, 1995) and this was also done in the ISC groups that were cooled. Cooling in the hypothermia groups (ISC+HYPO, SHAM+HYPO) was delayed until 12 h after reperfusion or sham occlusion (Colbourne et al., 1999). Hypothermia-treated gerbils were cooled slowly at a rate of 1 °C per 30 min to 33 °C (for 24 h) then slowly warmed at the same rate to 35 °C (for 24 h). At this time, gerbils were warmed to 36 °C and maintained between 36 and 37 °C for 12 h. Core telemetry probes were removed 9 days following ischemia under brief isoflourane anesthesia. Hypothermia was produced with an automated system that uses infrared lamps to heat and fine water mist and fans to cool (Colbourne et al., 1996).

Delayed Transient Ischemic Attacks

At 5 and 6 days following ischemia or sham surgery, all groups were further divided to receive a TIA (SHAM+NORMO+TIA, SHAM+HYPO+TIA, ISC+NORMO+TIA, ISC+HYPO+TIA) or sham surgery (SHAM+NORMO+SH, SHAM+HYPO+SH, ISC+NORMO+SH, ISC+HYPO+SH). All procedures were done as described above for ischemia; however, occlusion of the arteries in the TIA groups lasted 1.5 min on both days. The SH animals did not have their arteries occluded, but they were isolated. Brain temperature was measured and maintained at ~36.5 °C throughout the TIA/SH surgeries. These insults have been repeatedly used by others (Corbett and Crooks, 1997) to induce tolerance to subsequent lethal insults, but they do not kill CA1 neurons in naïve animals.

Behavioural Testing

At 4 days following ischemia or sham occlusion, gerbils were place in a novel maze (a black Plexiglas box with a transparent cover; 22-cm wide x 32-cm long x 15-cm in height) for 15 min, in a quiet room with overhead cues (Colbourne et al., 1998b). Movement activity, which is thought to reflect exploration in a novel environment (Colbourne et al., 1998b), was recorded during this test and was measured by the telemetry receiver's detection of variations in the core probe's signal strength. Testing on day four was done to confirm that TIA and SH subgroups of the SHAM+NORMO, SHAM+HYPO, ISC+NORMO, and ISC+HYPO groups were similar before TIA / SH intervention. Gerbils were exposed to a different and novel maze on day 9. Thus, any differences (or interactions) on this test day would then be due to the effects of the secondary TIAs. The data were statistically analyzed with ANOVA and Fisher L.S.D. tests.

On days 19 and 20, all groups were habituated to the T-maze (60-cm stem length, 30-cm arm lengths, 10-cm wide, 12-cm high) for three 5-min sessions/day (Colbourne et al., 1998b). During these sessions, gerbils had free access to both arms and half a sunflower seed reward in a food cup at the end of each arm. On days 21-26, gerbils were trained on a win-shift paradigm for sunflower seed reward. This consisted of 10 daily pairs of forced trials (FT) and choice trials (CT). Forced trials consisted of placing the gerbil in the start box of the maze and allowing it 60 s to enter an open goal arm of the maze (randomly chosen). Upon consuming the seed, animals are returned to the start box where they remain for 30 s. Following the FT, the gerbil is allowed to re-enter the maze (with both goal arms open) and has 60 s to choose. The correct response on the CT was

the arm opposite that on the FT. The % correct on the choice trial was analyzed by ANOVA.

Histology

Gerbils were sacrificed 30 days after ISC/SHAM operation with an overdose of sodium pentobarbital (0.1 ml) and perfused with saline followed by 10% neutral-buffered formalin. Brains were then processed, frozen, and 10-μm coronal sections were taken with a cryostat. Ischemic injury was quantified by counting the number of viable-looking (Cresyl violet stain) pyramidal neurons in the lateral (next to CA2), middle (apex of CA1), and medial (adjacent to subiculum) subsections of CA1 (each 0.2-mm long; 400X light microscopy) at –1.7 mm to bregma as previously done (Colbourne and Corbett, 1995). The experimenter conducting cell counts was blinded to the subjects' treatment. CA1 cell counts were analyzed with ANOVA followed by planned comparisons (Fisher L.S.D. test).

Results

Baseline core temperature did not differ among groups (Table 5.1). Brain temperature during the 5-min ischemic insult or sham occlusion and the 1.5 min TIAs or sham occlusions were also very similar among groups (Table 5.1). Core temperature following ischemia (Fig. 5.2) was regulated as described in Ischemia and Temperature control. There was no mortality associated with the treatments.

There were no significant differences among SHAM groups for CA1 cell counts in the medial ($F_{3,21}$= 1.583, P= 0.228), middle ($F_{3,21}$= 1.489, P=0.251) and lateral CA1 sectors ($F_{3,21}$<1), and thus these groups were combined for subsequent statistical analyses (likewise for behavioural tests; statistics not shown). In addition, an unblinded

examination of SHAM+TIA groups revealed no obvious signs of injury in CA1, nor in other hippocampal areas. In the ANOVAs that included the combined SHAM and all ISC groups there were significant group main effects for medial ($F_{4, 72}$=55.59, P<0.001), middle ($F_{4, 72}$= 50.667, P<0.001) and lateral CA1 sector cell counts ($F_{4, 72}$= 62.581, P<0.001). Extensive damage occurred in all sectors of CA1 in ISC+NORMO+SH and ISC+NORMO+TIA groups ($P < 0.001$ vs. SHAM; Figs. 5.3 and 5.4). However, these groups were not significantly different ($P \geq 0.586$) showing that delayed TIAs did not aggravate untreated ischemic injury, which was near maximal anyway. Hypothermia treatment markedly reduced CA1 injury in both ISC+HYPO+SH and ISC+HYPO+TIA groups ($P < 0.001$ for all sectors vs. ISC+NORMO groups); however protection was not perfect ($P < 0.001$ vs. SHAM). Moreover, these two groups differed significantly from each other in the medial sector ($P = 0.035$), near-significantly in the middle sector ($P =$ 0.052) and not significantly in the lateral ($P = 0.225$) sector. Delayed TIAs thus reduced hypothermic neuroprotection, but overall most neurons were well protected.

A one-way ANOVA on the day 4 maze data was significant ($F_{4, 64} = 6.532$, P< 0.001. The ISC+NORMO+SH and ISC+NORMO+TIA gerbils did not differ significantly from each other in the small maze on day 4 ($P = 0.715$) and both groups displayed significantly higher activity than SHAMs (P 0.002; Fig 5.5), which suggests a spatial habituation impairment. Both ISC+HYPO+SH and ISC+HYPO+TIA did not differ significantly from each other ($P = 0.410$). These hypothermia treated groups were significantly less active than ISC+NORMO groups ($P = 0.008$) and not significantly different from SHAM ($P \geq 0.427$), which suggests a normal habituation pattern. Thus, testing in the small maze on day 4 suggests untreated ischemic animals did not differ

from each other prior to secondary TIA. Statistical results of activity levels during the novel maze session on day 9 were similar (statistics not shown). Accordingly, ISC+NORMO groups were still impaired whereas ISC+HYPO groups were normal. The TIA insults did not worsen habituation ability.

An ANOVA on the average performance (percent correct; data not shown) in the T – maze did not yield an overall significant effect ($F_{4,64} = 2.106$, $P = 0.090$). Nonetheless, the ISC+NORMO groups (~76% success) were less successful (i.e. impaired working memory) that SHAMs (~82%) and the ISC+HYPO groups (~82%) were like SHAMs although they were not statistically better that ISC+NORMO groups. Removal of a few histological outliers did result in significant deficits of ISC+NORMO groups, but this did not affect the pattern of results; notably the TIAs did not worsen working memory performance in the T-maze.

Discussion

The primary findings of this study are that: i) some hypothermia-salvaged CA1 neurons are susceptible to delayed, normally sublethal, TIAs following hypothermic neuroprotection, and ii) many hypothermia-salvaged neurons are resilient to TIAs. Since secondary TIAs did not worsen CA1 cell death after untreated ischemia, it is likely that the secondary insults killed hypothermia-rescued cells in the ISC+HYPO+TIA group. In this study, hypothermia treatment was delayed until 12 h as this procedure provides and intermediate level of neuroprotection with a continuing, but partial, loss of CA1 neurons with time (Colbourne et al., 1999). In addition, some of the salvaged CA1 cells have residual morphological abnormalities (e.g., mitochondrial autolysosomes), which would be expected to compromise neuronal survival (e.g., impaired metabolism). Furthermore,

since 1-h delayed cooling does not fully prevent ischemia-induced downregulation of the AMPA channel GluR2 subunit (Grooms et al., 2000), one would expect a greater residual down-regulation with the 12-h delayed cooling. While these abnormalities likely contributed to sustained vulnerability in rescued CA1 cells, further studies are needed to prove the mechanism(s), which may also include but are not limited to; hemodynamic alterations (Danton et al., 2002), enhanced excitotoxicity (e.g. greater or more sustained elevations in glutamate; Lin et al., 1992), and alterations in gene expression. Additionally, future studies must examine the resiliency of cytoprotection in different brain regions (e.g., striatum) to various cellular stressors (e.g. brief local ischemia).

The reduction in CA1 survival because of delayed TIAs was significant in the medial CA1 zone. This is in agreement with data showing that postischemic hypothermia results in more persistent neuroprotection in middle and lateral CA1 sectors than in medial CA1 (Colbourne and Corbett, 1995). Less efficacious treatments, more severe or earlier secondary insults would likely affect all CA1 zones. The loss of neuroprotection in ischemic preconditioned animals given postischemic environmental enrichment (Farrell et al., 2001) was greater than that presently found with delayed TIAs after hypothermic neuroprotection even though both studies utilized a similar ischemia model. This could be due to the fact that ischemic preconditioning is less neuroprotective or that environmental enrichment was a more severe stress than TIAs, which also might be due to enrichment therapy being introduced 3 days after ischemia in that study (Farrell et al., 2001). Conversely, more effective therapies (e.g. intra-ischemic hypothermia, early and prolonged postischemic hypothermia) are less likely to be affected. Indeed, Colbourne and colleagues (Colbourne et al., 1998a), failed to lessen hypothermic neuroprotection

with a battery of behavioural tests starting on day 4 after ischemia. In that study, cooling was begun 6 h after ischemia and resulted in ~ 90 % protection of CA1.

In spite of TIAs reducing cell survival there were no obvious behavioural ramifications. Perhaps those additional CA1 neurons that died did not initially contribute to functional performance. For instance, they may have suffered from significant electrophysiological abnormalities. While an earlier study (Dong et al., 2001) found no such abnormalities in CA1 neuron rescued with postischemic hypothermia, it is important to note that protection was better in that study (early treatment intervention) and only a few neurons were examined making it impossible to generalize to all rescued neurons from various treatments. More demanding tests, such as the winstay variant of the T-maze (Colbourne and Corbett, 1995; Babcock and Graham-Goodwin, 1997) used by Farrell and colleagues (Farrell et al., 2001), might reveal behavioural effects. Additionally, such tests would be expected to show greater (and statistically significant) working memory deficits in untreated ischemic gerbils than that presently observed. In a previous study (Colbourne and Corbett, 1995), untreated ischemic gerbils were significantly impaired in the T-maze win-shift task. Perhaps the difference is due to total amount of hippocampal injury, which was somewhat less in this study, and/or the use of a different supplier in gerbils.

Interestingly, Farrell and colleagues (Farrell et al., 2001) observed improved functional outcome in an ischemic preconditioned group that was also given environmental enrichment, a group that had fewer surviving CA1 neurons than the preconditioned alone treatment. The effects of enrichment are thought to be due to a variety of cellular processes (e.g. sprouting) throughout the brain (Kolb et al., 1998).

Thus, various beneficial effects of enrichment outside of the CA1 zone and within surviving CA1 neurons could compensate for the increased loss of hippocampal CA1 neurons and it cannot be concluded that those lost neurons either had disturbed hippocampal function or would not have eventually contributed to proper hippocampal function.

Fever aggravates global ischemic injury when it occurs within the 1st day (Baena et al., 1997). We did not observe fever after the brief TIAs. However, it is possible that fever repeatedly accompanied the environmental enrichment paradigm used by Farrell et al. (2001). Animals commonly experience stress-induced fever with behavioural manipulations (e.g., small mazes, Colbourne et al., 1998b; present study- data not shown). Repeated changes in the enrichment cage (e.g. novel objects) might repeatedly cause stress-induced fever along with other physiological changes that could account for the exacerbated injury in that study. This would only occur in the ischemic preconditioned group and not in the untreated ischemic gerbils since it is the preconditioned groups that has salvaged and vulnerable CA1 neurons.

Aggressive motor rehabilitation (constraint-induced movement therapy) has also been shown to worsen cortical brain injury, when it is administered soon after the lesion (Kozlowski et al., 1996; Humm et al., 1998; Bland et al., 2000; DeBow et al., 2003). This effect seems to be dependent on glutamate (Schallert et al., 1997), and thus may share common mechanisms (use-dependency) as the lessening of CA1 neuroprotection in the present study. It is also possible that constraint-induced movement therapy aggravates brain injury by causing a localized rise in brain temperature in the vulnerable tissue (~ 0.7 °C elevation; DeBow et al., unpublished data). This possibility is being explored.

In summary, not all CA1 neurons salvaged by neuroprotective interventions are tolerant of delayed insults. Presently, some CA1 neurons were killed off by quite delayed TIAs. Accordingly, the clinical efficacy of treatments administered after global and focal ischemia might be undone by insults that would otherwise remain untreated. Such 'mild' insults may necessitate further therapy. Similarly, aggressive rehabilitation therapies may need to be delayed to avoid losing some previously saved tissue. Finally, the present paradigm may prove useful in determining whether cytoprotective strategies are truly resilient (i.e. permanent) as is mostly the case with prolonged cooling. This is supported by various experimental studies showing persistent protection with delayed hypothermia (Colbourne and Corbett, 1995; Colbourne et al., 2000) and recent clinical studies of hypothermia after cardiac arrest (Bernard et al., 2002; The Hypothermia After Cardiac Arrest Study Group, 2002).

Table 5.1

Mean core temperature (± S.D.) one day prior to ISC/SHAM operation and brain temperatures during ischemia/sham occlusion.

	Baseline Core temp. (C)	ISC/ SHAM brain temp. (C), occlusion	TIA/SH brain temp. (C), day 5	TIA/SH brain temp. (C), day 6
SHAM+NORMO+SH	37.6 ± 0.2	36.2 ± 0.7	36.3 ± 0.7	36.2 ± 0.3
SHAM+NORMO+TIA	37.4 ± 0.3	36.4 ± 0.5	35.8 ± 0.5	36.2 ± 1.0
SHAM+HYPO+SH	37.5 ± 0.3	36.2 ± 0.5	36.2 ± 0.4	36.3 ± 0.5
SHAM+HYPO+TIA	37.5 ± 0.4	36.2 ± 0.4	35.5 ± 1.0	36.0 ± 0.6
ISC+NORMO+SH	37.4 ± 0.3	36.1 ± 0.6	36.3 ± 0. 5	36.3 ± 0.4
ISC+NORMO+TIA	37.5 ± 0.2	35.9 ± 0.4	35.9 ± 0.5	35.8 ± 0.4
ISC+HYPO+SH	37.5 ± 0.3	35.9 ± 0.6	35.9 ± 0.6	36.2 ± 0.4
ISC+HYPO+TIA	37.4 ± 0.3	35.9 ± 0.5	36.3 ± 0.5	36.4 ± 0.5

Figure 5.1

Diagram illustrating treatment groups and numbers. Animals were randomly assigned to receive SHAM surgery or ISC (5-min occlusion). Then 12 h after surgery, hypothermia treatment began in half of the animals, and the other half was regulated as described in Ischemia and Temperature Control. At 5 and 6 days following ISC and SHAM surgery, animals were again randomly assigned to receive a 1.5-min TIA or SH insult on each day.

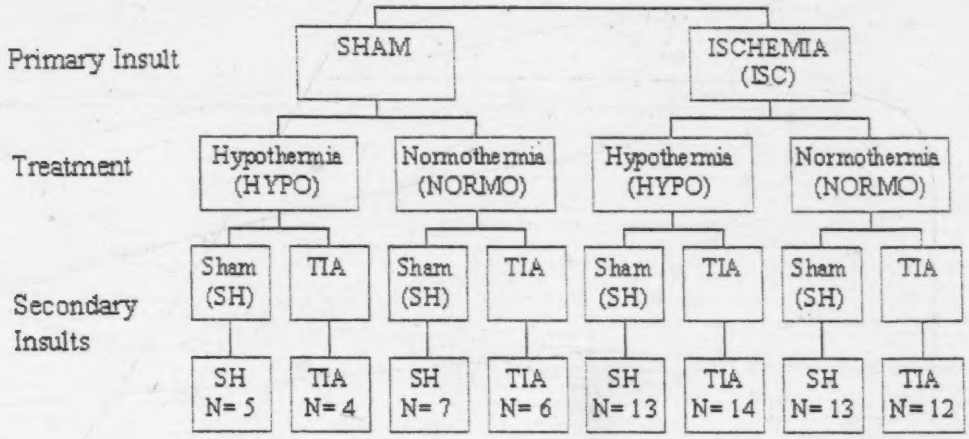

Primary Insult
SHAM
ISCHEMIA (ISC)
Treatment
Hypothermia (HYPO)
Normothermia (NORMO)
Hypothermia (HYPO)
Normothermia (NORMO)
Secondary Insults
Sham (SH)
TIA
Sham (SH)
TIA
Sham (SH)
TIA
Sham (SH)
TIA
SH N= 5
TIA N= 4
SH N= 7
TIA N= 6
SH N= 13
TIA N= 14
SH N= 13
TIA N= 12

Figure 5.2

Core temperature averaged every 5 minutes (sampled every 30 seconds) beginning immediately following ISC or SHAM surgery until 4 days later. Temperature profiles beyond this time were very similar and thus not shown. Temperature following ischemia was regulated as described in Ischemia and Temperature Control. See Table 5.1 for mean brain temperature values.

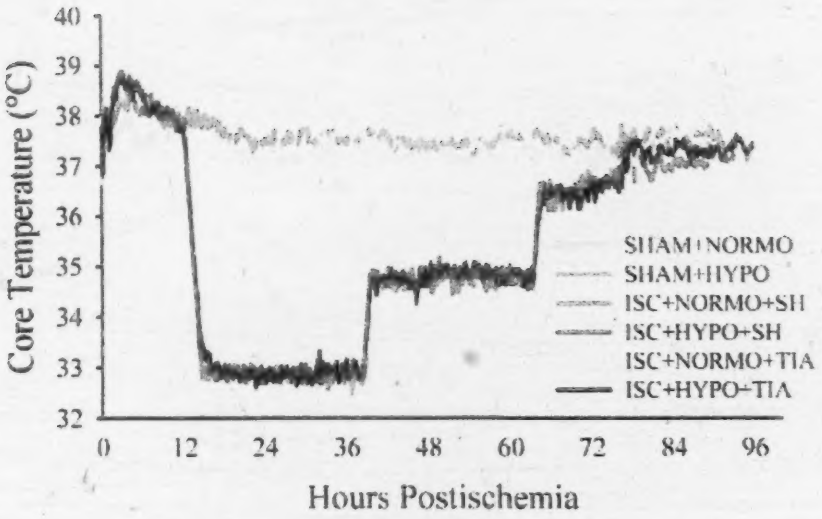

Core Temperature (°C)
40
39
38
37
36
35
34
33
32
0
12
24
36
48
60
72
84
96
Hours Postischemia
SHAM+NORMO
SHAM+HYPO
ISC+NORMO+SH
ISC+HYPO+SH
ISC+NORMO+TIA
ISC+HYPO+TIA

Figure 5.3

CA1 cell counts expressed as a mean percentage of SHAM (± S.D.). SHAM groups were not significantly different from each other. Cell counts in the untreated ischemia groups (ISC+NORMO+SH/TIA) were not significantly different. However, the ISC+HYPO+TIA had significantly fewer remaining neurons in the medial CA1 with similar trends in middle and lateral CA1. See Fig. 5.4 for representative photomicrographs.

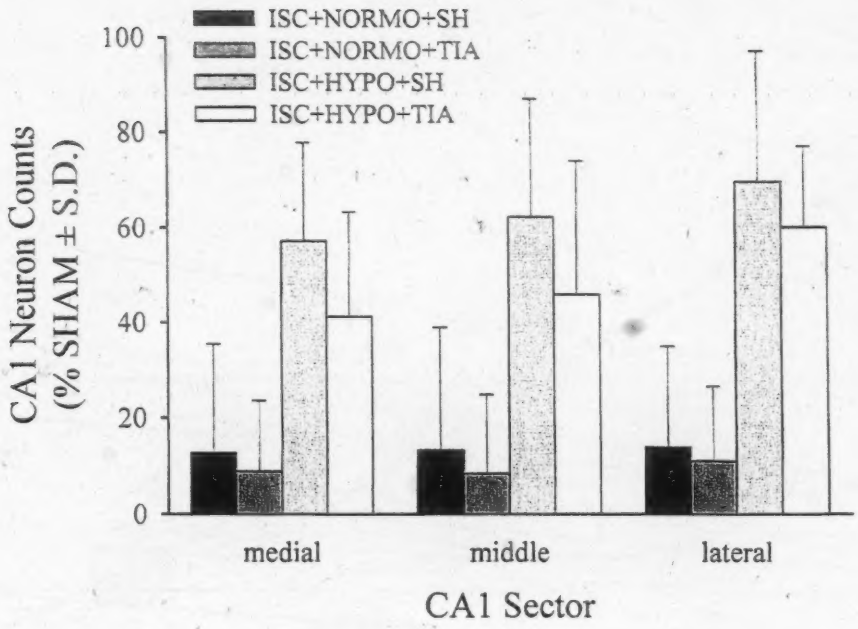

ISC+NORMO+SH
ISC+NORMO+TIA
ISC+HYPO+SH
ISC+HYPO+TIA
CA1 Neuron Counts
(% SHAM ± S.D.)
100
80
60
40
20
0
medial
middle
lateral
CA1 Sector

Figure 5.4

Representative photomicrographs of medial CA1 in SHAM+NORMO+SH (A), ISC+HYPO+SH (B), ISC+HYPO+SH (C), and ISC+HYPO+TIA (D) groups (scale bar represents 100 μm). See Colbourne et al., 1999 for electron micrographs of untreated and hypothermia treated ischemia CA1 injury.

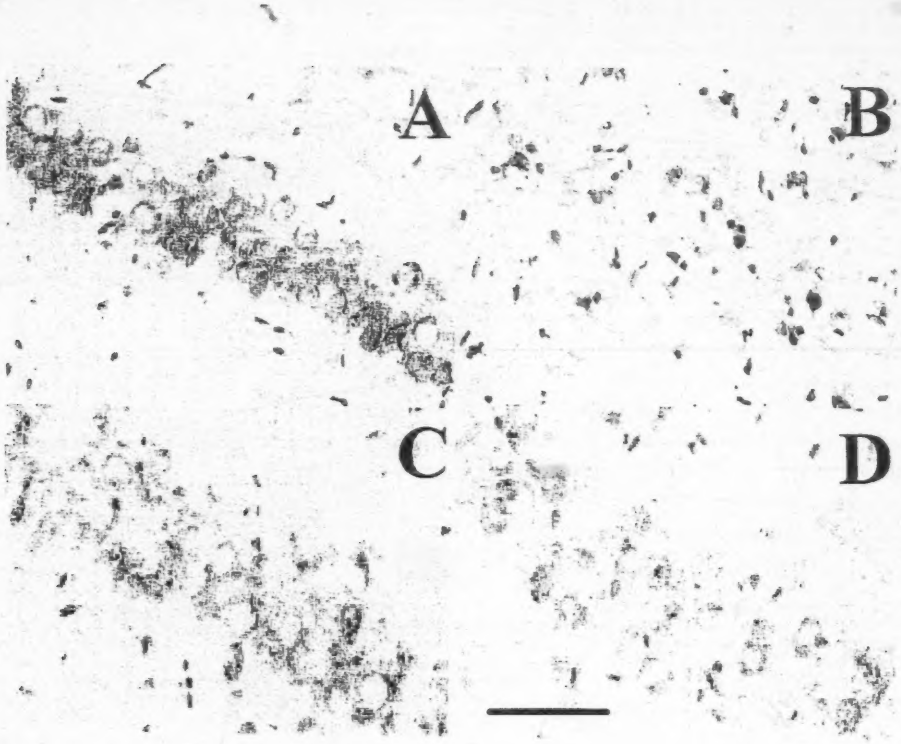
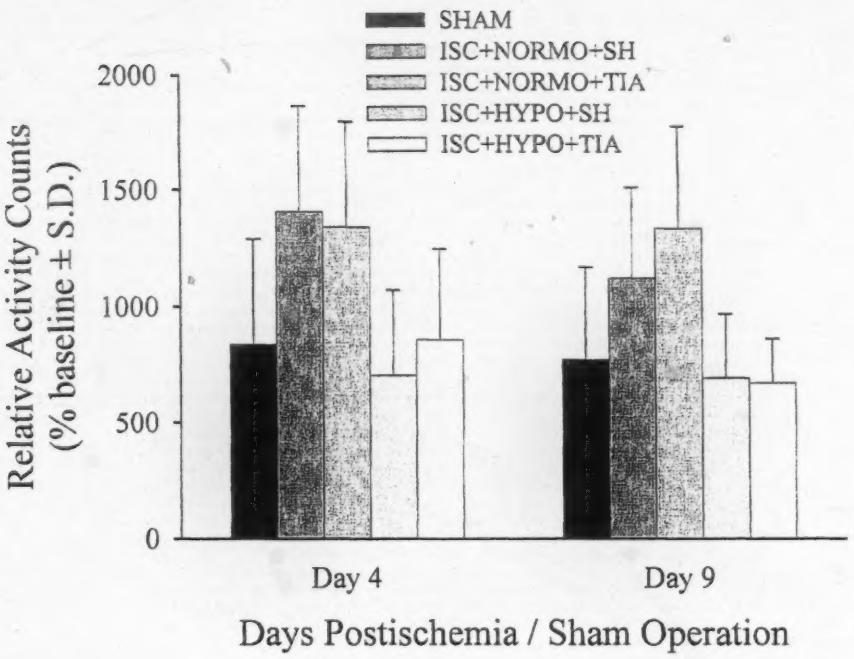

A
B
C
D

Figure 5.5

Mean activity counts (sampled every 30 s and averaged) measured with the core telemetry probe in the small mazes on days 4 and 9 following ISC/SHAM surgery. On day 4 both ISC+NORMO+TIA/SH groups were significantly hyperactive compared with SHAMs, and both ISC+HYPO groups. The TIA or SH interventions occurred on days 5 and 6. On day 9 ISC+NORMO+TIA/SH groups were significantly hyperactive compared to SHAMs and ISC+HYPO+TIA/SH groups. The TIA treatments did not affect activity levels.


SHAM
ISC+NORMO+SH
ISC+NORMO+TIA
ISC+HYPO+SH
ISC+HYPO+TIA
Relative Activity Counts
(% baseline ± S.D.)
2000
1500
1000
500
0
Day 4
Day 9
Days Postischemia / Sham Operation

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Chapter 6

Constraint-induced movement therapy immediately following ischemia causes a localized hyperthermia that exacerbates injury

Hemiparesis of the upper limb after stroke often necessitates intense rehabilitation and long-term care for survivors (Taub et al., 1993). Fine movements of the impaired limb become difficult to perform. Consequently, stroke survivors become accustomed to performing daily tasks with only their good limb, and cease any attempt at using their impaired side, a phenomenon known as "learned non-use" (Taub et al., 1993). Although traditional rehabilitation (e.g., encouraging compensatory strategies with the good limb) is administered to stroke patients, little or no functional recovery of the impaired side is observed (Kalra, 1994). Constraint-induced movement therapy (CIMT) attempts to overcome "learned non-use" by restraining the good limb (e.g., use of a sling) thereby forcing use of the impaired (Taub et al., 1993). In chronically impaired stroke patients (e.g., one year post-stroke), CIMT administered daily for two weeks provided lasting improvements of motor function that transferred into the everyday life of the patient (Ostendorf and Wolf, 1981; Wolf et al., 1989; Miltner et al., 1999). Thus, the question arises whether CIMT may provide further benefit if it were administered *soon* after injury, perhaps even prior to the development of the learned non-use.

In rodents, unilateral injury to the forelimb representation area of the sensorimotor cortex results in deficits comparable to human stroke, and is used to examine mechanisms underlying CIMT. However, when applied too soon after various types of injury, CIMT exacerbates damage. For instance, CIMT immediately following electrolytic lesions (Kozlowski et al., 1996; Humm et al., 1998), and middle cerebral artery occlusion (Risedal et al., 1999; Bland et al., 2000) *worsens* brain injury and slows behavioural recovery. Delaying the onset of CIMT for seven days prevents exacerbation of injury following electrolytic lesions (Humm et al., 1998), and promotes both functional and

neurological recovery following intrastriatal hemorrhage in rats (DeBow et al., 2003). The implication that CIMT early after injury could worsen outcome raises clinical concerns, as activation and training programs begin early after stroke in most stroke centers (Hamrin, 1982).

A number of possible mechanisms for CIMT-induced exaggeration of injury exist. First, immobilization of the unimpaired limb causes a forced over-reliance upon the impaired limb immediately following injury (Kozlowski et al., 1996). This over-reliance may cause increased depolarization of cortical cells (e.g., glutamatergic neurons) in the injured cortex resulting in exposure of neurons to toxic concentrations of glutamate (Kozlowski et al., 1996). Alternatively, CIMT itself may cause a systemic side effect that aggravates injury (e.g., fever and/or stress). For instance, the CIMT procedure itself may cause a rise in whole body temperature or an increase in glucocorticoids, which are both detrimental to ischemic outcome (Sapolsky, 1992). Finally, perhaps a combination of one or more of these mechanisms is involved in CIMT-induced potentiation of injury. Post-ischemic concentrations of extracellular glutamate have profound effects on recovery after ischemic injury, and are affected by temperature (Asai et al., 2000). For instance, intra-ischemic hyperthermia inhibits re-uptake of extracellular glutamate during the postischemic period resulting in prolonged exposure of neurons to toxic concentrations of glutamate (Asai et al., 2000). Previous studies examining CIMT following electrolytic lesions of the sensorimotor cortex (Kozlowski et al., 1996) and middle cerebral artery occlusion (Bland et al., 2000) report no temperature change in casted animals. However, large temperature changes can occur within relatively short time periods (Corbett and Thornhill, 2000), and may go unnoticed with intermittent (i.e., once a day) temperature

measurement. Furthermore, local changes in brain temperature can occur during behaviour and in response to various novel, stressful (e.g., CIMT) and emotionally arousing stimuli (Kiyatkin et al., 2002). Therefore, this study was done to characterize the effects of CIMT on core and brain temperature following a devascularization model in the rat. This model involves removal of the leptomeninges from a specific cortical region (e.g., forelimb representation of the sensorimotor cortex). This lesion was chosen since two lesions can be produced in the same rat. In the first experiment, core temperature was measured beginning immediately after injury for seven days. Brain temperature was measured (experiment 2) as well as controlled (experiment 3) immediately following injury for at least four days. We hypothesize that CIMT (in rats forced to use their impaired limb) will cause either a systemic (e.g., whole body) and/or a localized (e.g., motor cortex region) increase in temperature.

Materials and Methods

Subjects

All rats were group housed until surgery, and maintained on a 12-hour light dark cycle with access to food and water. All procedures followed the Canadian Council for Animal Care guidelines and were approved by local animal care committees.

Experiment 1

We hypothesized CIMT in rats forced to use their impaired limb will induce a systemic (e.g., whole body) increase in temperature. In order to test this possibility, we examined whether an over-reliance on the impaired limb due to CIMT (i.e., overactivation of injured cortical neurons) or a systemic side effect (e.g., fever) causes exacerbation of brain injury. Two unilateral right hemisphere lesions of equal size (4

mm^2) were made, one in the motor cortex and one in the visual cortex of the same rat. If CIMT-induced worsening of injury is via excessive neuronal activation due to increased behavioural pressures, then only the motor cortex lesion should increase in size, as movement of the forelimb would not cause cell death of neurons in the visual cortex. However, if CIMT causes a systemic side effect, such as whole body hyperthermia, then both motor and visual cortex lesions should increase in rats forced to use their impaired limb.

Surgery

Twenty-six male Long-Evans rats (250-350g, University of Lethbridge, AB, CAN) under isoflourane gas (in 30% O_2 and 70% N_2O) received two 4 mm^2 craniotomies and devascularization lesions in the same hemisphere. The first was above the forelimb area of the sensorimotor cortex (1.5 mm AP, 2.0 mm ML to bregma marked the front left corner), and the second was above the visual cortex (-6.0 mm AP and 1.5 mm ML to bregma). Devascularization lesions were made by removing the leptomeninges from the surface of the cerebral cortex using fine iris scissors (Fine Science Tools, North Vancouver, Canada) followed by the lowering (1 mm ventral from the surface of the skull) of a 4 mm^2 box-shaped blade into the cortex to interrupt arterial blood flow. We used the devascularization model in order to produce two equivalent stroke-like lesions in the same rat, which was not possible with traditional occlusive stroke models (e.g., suture). Two of the twenty-six rats were excluded, one due to excess bleeding during surgery, and one due to self-removal of the cast prior to the end the experiment.

Four days prior to devascularization surgery in a subset of animals (N = 4), core telemetry probes (model TA1OTA-F40; Data Sciences, St. Paul, MN, USA) were

implanted into the peritoneal cavity. Temperature was continually sampled every 30 s and recorded for seven consecutive days following injury using an automated system (DQ3 System, DataSciences, St Paul, MN, USA; previously described in detail in (Colbourne, 1999). These rats received lesions to the motor cortex only.

CIMT Procedure

Immediately after surgery, all rats were randomly assigned to receive either ipsilateral cast (non-impaired forelimb was restrained; IPSI; N = 10) or contralateral cast (limb contralateral to the lesion was restrained; CONTRA; N = 10). A Plaster of Paris cast that remained in place for 24 hours a day for 7 days a week, was wrapped around the upper torso and the desired limb of each rat to form a one-sleeved jacket (Jones and Schallert, 1994; Kozlowski et al., 1996). Rats in the IPSI group were forced to use their impaired limb, while CONTRA rats used their non-impaired limb for feeding, grooming and walking during the casting period.

Experiment 2

We examined whether CIMT immediately following injury causes an increase in brain temperature (when measured at ~1 mm anterior to sensorimotor cortex lesions). Brain temperature was measured every 30 seconds with the ART 2.2 automated system (DataSciences; Colbourne et al., 1996; DeBow and Colbourne, 2003).

Brain Probe Implantation

In order to measure brain temperature, a novel method was developed allowing placement of the brain telemetry probe (model VM-FH, Mini-mitter Co. Inc, Bend, Oregon USA; Fig 1) directly into the tissue surrounding injury (~ 0.5 -1 mm anterior from the edge of the box cut). One day prior to surgery a 20 gauge guide cannula (5 mm

in length, Plastics One, Roanoke, VA, USA), was secured with dental cement and three metal screws to the centre of a 1 cm long plastic tube (~ 1 cm of a 3 cc syringe, Fig 6.1B). The tube protected the brain probe (Fig 6.1A) and prevented damage from the rats' daily activities (e.g., grooming, sleeping).

Four days prior to devascularization surgery, a 4 mm^2 craniotomy above the forelimb area of the sensorimotor cortex was made and the brain probe was inserted into the tissue. The brain probe was secured to the rats' skull with three small screws around the base of the syringe. A small portion of the headcap molded prior to surgery with dental cement formed a 4 mm^2 protrusion extending 1 mm from the surface of the skull to replace the bone (Fig 1). Therefore, only the shaft of the brain probe descended into the cortex (2 mm from the surface of the skull).

Surgery

Twenty-six male Wistar rats (250-350g; Ellerslie, AB, Canada) were used. On the day of surgery, all rats were anesthetized with isoflourane gas (in 30% O_2 and 70% N_2O). A 4-mm^2 craniotomy and devascularization lesion of the motor cortex were done as described in experiment one (visual cortex lesions were not made in this experiment). Immediately following injury, the headcap and brain telemetry probe were re-affixed to the skull, and remained in place throughout the casting treatment (seven days). Five rats were excluded due to excessive bleeding during surgery. Two rats were removed due to premature headcap removal leaving a total of 19 rats in this experiment.

CIMT procedure

Immediately following the devascularization lesion rats were randomly assigned to receive either IPSI (N = 10) or CONTRA (N = 9) cast. For experiment 2 however, a

tensor bandage (Coban, 3M™ , London, Ont., Can) was wrapped around the abdomen and appropriate limb of each rat in lieu of the Plaster of Paris cast used in experiment 1. The tensor bandage method was used to reduce the risk of damage to the brain probe, as it is significantly lighter and more flexible.

Experiment 3

We hypothesize that forced hyperthermia in rats allowed to use their good limb (CONTRA) will result in aggravation of injury similar to that previously observed in IPSI rats. In order to test this hypothesis, brain temperature was forcibly increased in CONTRA casted rats (CONTRA-Reg) using the brain probe implantation method described in experiment 2 and a telemetry feedback system and heating lamps. The post-ischemic temperature changes used to externally increase temperature of these rats were based upon temperature data for IPSI rats in experiment 2.

Surgery

All rats received devascularization of the motor cortex and headcap placement as previously described in experiment 2. Following surgery, all rats were randomly assigned to receive either IPSI cast (N = 13), CONTRA cast (N = 12) with no temperature regulation or CONTRA cast with temperature regulation (CONTRA-Reg; N = 16). Casting was done with the same tensor bandage method used in experiment two. Brain temperature was regulated in the CONTRA-Reg for the first 24 hours following injury. Temperature was elevated 1°C (36.0 °C to 37.0 °C) over the first 5 hours post-injury, and then decreased to 36.5°C over19 hours using infrared heat lamps (175-250 W) and an automated feedback system (ART 2.2., DataSciences; Colbourne, 1996; DeBow, 2003).

Brain probes were removed four days after devascularization to prevent damage to the probe.

Histology

All rats were killed sixty days following devascularization surgery with sodium pentobarbital (80 mg/kg) and then perfused with saline followed by 10% formalin. Brains were processed, and 40-μm (every 200 μm) coronal sections were taken with a cryostat and stained with cresyl violet (DeBow et al, stroke, 2003). The total size of lesion was quantified using Scion Image J 4.0 (Scion Corporation), and expressed as: (Remaining Tissue of Lesioned Hemisphere/ Remaining Tissue of Non-Lesioned Hemisphere)*100.

Statistical Analysis

Lesion size was assessed using ANOVA and Fisher L.S.D. post hoc test (experiment 3 only). Post-ischemic brain and core temperature recordings (30 s samples averaged over 5 minutes for 24 h) were analyzed using ANOVA (experiment 1 and 2) and Fisher L.S.D post hoc test (experiment 3). All variance terms presented are standard deviation (SD).

Results

Experiment 1

Two months following injury the IPSI cast group had significantly larger motor cortex lesion (91.83% ± 4.3 SD) than the CONTRA cast group (96.03% ± 2.2; p = 0.026). Visual cortex lesions did not differ significantly in size between IPSI (93.6% ± 4.4) and CONTRA groups (91.5 ± 3.3; $p = 0.308$). Core temperature also did not differ significantly between IPSI and CONTRA rats ($p = 0.193$, Fig. 2A).

Experiment 2

The IPSI cast group had significantly larger lesions (92.6% ± 3.7) compared to CONTRA cast group (96.7% ± 4.3; $p = 0.044$). Brain temperature measurements revealed that the IPSI cast group had significantly elevated brain temperature compared to CONTRA cast group for the first 24 hours following injury ($p = 0.004$; Fig. 2B).

Experiment 3

As before, we found IPSI cast rats had significantly larger brain injury (83.0% ± 2.0) compared with CONTRA cast rats (85.6% ± 1.9; $p = 0.016$). Further, CONTRA-Reg rats also had significantly larger lesions (82.7% ± 3.3) compared to CONTRA rats ($p = 0.006$), but did not differ significantly from IPSI rats ($p = 0.758$). Temperature profiles for the first 24 hours postischemia of CONTRA cast rats (36.5° ± 0.3) were significantly lower than CONTRA-Reg rats (37.0°C ± 0.2; $p = 0.031$) and near significance compared to IPSI cast (37.0° ± 0.3; $p = 0.068$).

Discussion

Early CIMT (IPSI group) worsened ischemic injury in all three experiments. This effect is apparently not due to a systemic side effect (e.g. increased steroid levels) since only motor cortex lesions and not visual cortex lesions were exacerbated in experiment one. Moreover, it appears that aggravation of injury was also not due to a whole body hyperthermia as previously postulated (Colbourne et al., 1998), since no core temperature differences were detected between groups. A localized hyperthermia (~ 1°C) however, was observed in rats forced to use their impaired limb (IPSI cast) beginning immediately after the insult, and lasting for ~ 24 hours. Taken together, these findings support previous reports that immediate forced use of the impaired limb exacerbates cortical

injury and does not cause an overall change in body temperature or corticosterone levels (Kozlowski et al., 1996; Bland et al., 2000).

Small increases in temperature are sufficient to worsen neurological outcome (Ginsberg and Busto, 1998). Presumably, localized hyperthermia occurred in IPSI rats due to increased behavioural use of the impaired limb. Immediately following unilateral injury, rats depend on their good limb as a "crutch" to perform weight-shifting movements during normal exploration (Schallert et al., 1997). This compensatory strategy is followed by activity-dependent increases in dendritic arborization and neuronal overgrowth in the intact hemisphere and can be blocked by preventing use of the good limb (Jones and Schallert, 1994). It is therefore likely that forcing rats to use their impaired limb also causes increased neuronal activity (e.g., depolarization of glutamatergic neurons) in the injured cortex. Neural activity (e.g., in response to movement of the impaired limb) is metabolically expensive, and is accompanied by heat release in activated brain regions (Kiyatkin et al., 2002). Thus, this increased demand on cortical neurons due to CIMT likely resulted in the observed localized hyperthermia and caused susceptible neurons surrounding the initial lesion (e.g., penumbral neurons) to be recruited into the infarct core.

This possibility prolonged neuronal vulnerability is supported by studies examining other forms of behavioural manipulation (e.g., environmental enrichment; EE). When applied immediately after middle cerebral artery occlusion, EE aggravates neurological outcome (Risedal et al., 1999). However, delaying the onset of EE until seven days after injury prevents the enlargement of lesion size (Risedal et al., 1999). Overall, these data emphasize the possibility that intensive behavioural pressures (e.g.,

CIMT) too soon after injury may worsen both neurological and perhaps behavioural recovery. It is also possible that neurons previously salvaged with cytoprotective agents (e.g., post-ischemic hypothermia) will survive injury, but remain susceptible to secondary insult. For instance, hippocampal CA1 neurons previously salvaged with ischemic preconditioning were susceptible to EE when it was applied early (e.g., 3 days after injury) after injury (Farrell et al., 2001). Furthermore, we have previously shown CA1 ischemic neurons salvaged with post-ischemic hypothermia are vulnerable to secondary sub-lethal transient ischemic attacks (DeBow and Colbourne, 2003). In summary, these results indicate that intensive behavioural intervention *soon* after injury may cause deleterious effects not only to injured neurons left untreated, but also more importantly to cells that have previously been salvaged with neuroprotective strategies.

The substantial rise in extracellular glutamate due to neuronal excitation after ischemia is often implicated in the secondary processes leading to neuronal death (Choi et al., 1987; Choi, 1988; Globus et al., 1990). Moreover, brain regions involved in motor function are susceptible to excitation due to the relatively high concentrations of glutamate receptors (e.g., *N*-methyl-D-aspartate; Monaghan and Cotman, 1985). In casted animals, dialysate levels of glutamate were higher when sampled from the sensorimotor cortex contralateral to the free limb compared to the ipsilateral side (Bland et al., 1999), suggesting a potential role of glutamate in exacerbation of injury. In fact, 1 mg/kg of MK-801 (an NMDA antagonist) prevented CIMT-induced exacerbation of injury (Humm et al., 1999). However, in that study, MK-801 failed to attenuate injury in non-casted rats that presumably used only their good limb, suggesting other mechanisms (e.g., temperature fluctuations) may also be involved. Perhaps MK-801, which causes

significant post-ischemic hypothermia (Buchan and Pulsinelli, 1990), attenuated injury by blocking CIMT-induced localized hyperthermia. It is plausible therefore, that a combination of both hyperthermia and excitotoxicity (i.e., due to increased levels of extracellular glutamate) are involved in CIMT worsening of injury.

As hypothesized, we found that forcibly increasing brain temperature caused significantly larger injury in the CONTRA-Reg group compared to the unregulated CONTRA group. As the impaired limb was immobilized in both groups, these data show that hyperthermia on its own, and not in combination with forced use of the impaired limb, is sufficient to cause exacerbation of injury. Future studies examining the effects of blocking hyperthermia during forced use are needed in order to further characterize the role of temperature during rehabilitation. Perhaps, blocking localized hyperthermia during CIMT may prevent exacerbation of injury, and promote functional recovery. However, it is possible that activity of the limb itself is causing the hyperthermia. Activity-dependent increases in core body temperature are known to occur during exercise in normal rats (Fruth and Gisolfi, 1983), as well as due to limb movement during casting in ischemic rats (unpublished data). Thus, future studies are also needed to discern the source of the localized hyperthermia observed during CIMT.

Interestingly, the size of injury (i.e., percent damaged) was greater in the last experiment than in the previous two (in all groups). This may, in part, be due to early removal of the headcap and brain probe (e.g., 4 days in exp. 3 vs. 7 days in exp. 2). A new headcap method was developed for this study that allowed the direct measurement of brain temperature with telemetry probes from the site of injury. With this method, continual sampling of brain temperature every 30 s was measured beginning immediately

after injury. In addition, the brain probe assembly was constructed in such a way that it replaced the skull bone removed during the craniotomy, thereby preventing swelling of brain tissue through the craniotomy as sometimes seen with lesions of the motor cortex (Whishaw, 2000). This method is not only useful because it can be assembled prior to surgery, but also because it can easily be removed and replaced if need be throughout the experiment. Perhaps removing the headcap, and thus exposing the injured cortical tissue beneath the craniotomy four days after injury resulted in further injury and progression of injury. Craniotomy size and location are critical in determining extent of tissue injury produced during brain injury in rats (Vink et al., 2001). Nevertheless, IPSI cast had enlarged injury compared to the CONTRA group, confirming evidence of early CIMT causing injury.

The effects of CIMT have also been assessed in other animal models of cerebral injury. For instance, recent studies have examined the use of CIMT in rodent models of Parkinson disease (Tillerson et al., 2001; Tillerson et al., 2002). Patients suffering from Parkinson disease (PD) show learned compensatory behaviours similar to those observed in stroke patients. Likewise, rats with unilateral depletion of striatal dopamine, an animal model of PD, show preferential use of their non-impaired limb. Physical therapy can enhance motor ability in PD, and is thought to slow neuronal degeneration (Toole et al., 2000). In fact, when CIMT is applied to rats with unilateral 6-hydroxy dopamine lesions, behavioural improvements of the impaired paw, as well as sparing of striatal dopamine in the injured hemisphere was observed (Tillerson et al., 2001). Rats prevented from using their impaired limb immediately after injury suffer severe functional deficits and greater loss of dopamine terminals (Tillerson et al., 2002). Thus, contrary to results of CIMT

after stroke, forced use of the impaired not only provides functional recovery, but also prevents neuronal injury. In addition, CIMT provides both functional and neurological recovery following intracranial hemorrhage via injections of bacterial collagenase into the striatum (DeBow et al., 2003). It appears therefore, that the effects of CIMT may depend on the location of injury (e.g., subcortical vs. cortical). Both PD and ICH are subcortical injuries, whereas previous studies showing CIMT-induced aggravation have involved cortical injury (Kozlowski et al., 1996; Humm et al., 1998; Bland et al., 2000). This conflicting data may, in part, be due to differences in neuronal susceptibility to secondary stress following injury and/or to the type of injury.

In summary, these data show that that CIMT initiated *too soon* after injury worsens histological outcome in a model of devascularization, in agreement with previous findings in other rodent models of cerebral ischemia (Kozlowski et al., 1996; Humm et al., 1998; Risedal et al., 1999). It is clear that a critical period exists in the post-lesion period when vulnerable neurons that have survived the primary insult are susceptible to secondary injury. It is likely that potential factors resulting in secondary injury include both hyperthermia and increased behavioural demand (e.g., neuronal over-excitation) on injured cortex. Perhaps delaying CIMT (e.g., seven days post-injury) prevents exacerbation of injury and affords recovery because it is administered beyond this critical period. Further studies are needed however, to elucidate the exact role of secondary stressors and attempt to prevent them. Clinically, early CIMT may be possible without neurological consequences, if properly monitored and controlled. Traditional rehabilitation strategies applied early following stroke provide the most functional recovery (Dombovy et al., 1986; Kwakkel et al., 1997), while less is observed at 4 to 5

months, and almost no functional improvement is expected after 6 months (Andrews et al., 1981). These findings emphasize the need for early administration of rehabilitation strategies such as CIMT to provide maximum benefit to stroke patients. The fact that CIMT-induced aggravation of injury has now been demonstrated in various rodent models, strongly emphasizes the need to be cautious when administering any rehabilitation strategies during this critical period. Future studies characterizing underlying mechanisms, and attempting to prevent CIMT-induced exacerbation are warranted. We must define this critical period, which undoubtedly depends upon the type and location of injury.

Figure 6.1

Headcap assembly used to measure temperature continually in all casted rats. Brain telemetry probe (A; model XM-FH, Mini-mitter Co. Inc, USA) is inserted into a 3 cc syringe via a 20 gauge guide cannula (B). Brain probe shaft protrudes 2 mm below the cannula into the cortex (A), and is secured to the skull with by 3 small screws (B).

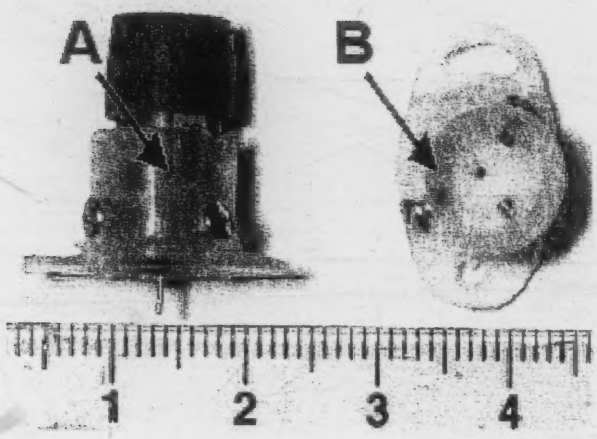
A
B
1
2
3
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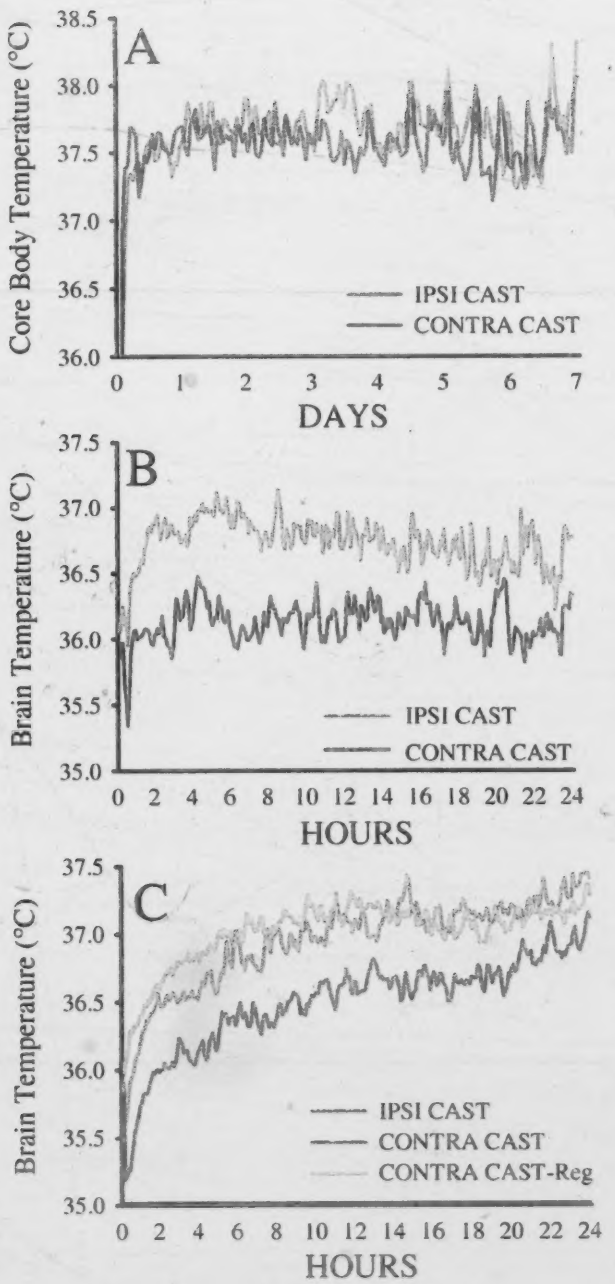
Figure 6.2

Post-ischemic temperature measured with core or brain telemetry probes. No difference in core temperature between IPSI and CONTRA cast rats ($p = 0.193$) was found for 7 days following injury (experiment 1, **A**). Brain temperature was significantly higher in IPSI cast rats compared with CONTRA cast rats ($p = 0.004$, **B**) for 24 h following injury. IPSI rats had higher brain temperature than CONTRA rats for 24 h, which nearly reached significance ($p = 0.068$, **C**). Brain temperature in CONTRA-Reg rats was forcibly elevated above CONTRA rats ($p = 0.031$), and did not differ significantly from IPSI cast rats ($p = 0.784$, **C**).


A
Core Body Temperature (°C)
38.5
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37.0
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36.0
IPSI CAST
CONTRA CAST
0 1 2 3 4 5 6 7
DAYS
B
Brain Temperature (°C)
37.5
37.0
36.5
36.0
35.5
35.0
IPSI CAST
CONTRA CAST
0 2 4 6 8 10 12 14 16 18 20 22 24
HOURS
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Brain Temperature (°C)
37.5
37.0
36.5
36.0
35.5
35.0
IPSI CAST
CONTRA CAST
CONTRA CAST-Reg
0 2 4 6 8 10 12 14 16 18 20 22 24
HOURS

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Chapter 7

Constraint-induced movement therapy and rehabilitation exercises lessen motor deficits and volume of brain injury after striatal hemorrhagic stroke in rats

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Hemiparesis affects the majority of stroke survivors acutely. While some spontaneous recovery occurs, many are chronically impaired (Jorgensen et al., 1999), in part because of "learned non-use" (Taub and Morris, 2001). This results from repeated failure with the impaired limb soon after injury combined with successful use of the normal limb. Restraint of the unimpaired limb (e.g., via sling for 6 h/d for 2 weeks) to force the use of an impaired limb during normal daily activities and rehabilitation exercises counteracts learned non-use. This therapy, known as constraint-induced movement therapy (CIMT), persistently decreases motor deficits after stroke in humans (Wolf et al., 1989; Miltner et al., 1999; van der Lee et al., 1999; Dromerick et al., 2000).

Approximately 20% to 25% of ischemic stroke survivors could benefit from CIMT (Blanton and Wolf, 1999). Unfortunately, there are few clinical and no experimental data on the efficacy of CIMT after intracerebral hemorrhage (ICH). For instance, data from 2 hemorrhagic patients, discontinued from traditional therapy, showed that CIMT lessened hemiparesis (Levy et al., 2001). Further study is warranted especially because rodent studies show that early CIMT may worsen brain injury and depress recovery. In rats, CIMT initiated immediately after cortical injury produced by electrolytic lesions (Kozlowski et al., 1996; Humm et al., 1998) middle cerebral artery occlusion (Bland et al., 2000), and cortical devascularization (unpublished data) results in enlarged lesions and poorer recovery. This is thought to result from heightened behavioural pressure (ie, neuronal overactivity) forced on vulnerable tissue (Kozlowski et al., 1996). Thus, given the trend toward beginning rehabilitation soon after stroke (Kwakkel et al., 1997), early CIMT might worsen occlusive and hemorrhagic stroke

outcome. Although preliminary data6 indicate that CIMT begun within 4 to 14 days after stroke in humans does not worsen outcome, hemorrhagic stroke patients were excluded.

We tested whether the combination of CIMT and rehabilitation exercises or each alone would improve motor recovery in rats after ICH (Rosenberg et al., 1990; Del Bigio et al., 1996). Therapies began 1 week after surgery and lasted for 7 days. Motor recovery on several tests was assessed over 2 months, and the volume of brain tissue destruction was quantified at 2 months.

Materials and Methods

Animals

Sixty-seven male Sprague-Dawley rats (weight, 250 to 350 g; obtained locally) were individually housed with food and water ad libitum. Procedures were approved by the Biosciences Animal Policy and Welfare Committee of the University of Alberta and in accordance with the Canadian Council on Animal Care guidelines. Figure 7.1 summarizes procedures, and Table 7.1 indicates group sizes.

Behaviour Training

Montoya Staircase Task

Rats were food deprived to 85% of their free-feeding weight 3 days before training (MacLellan et al., 2002). Training in the test, which measures independent forelimb reaching ability, consisted of two 15-minute trials per day separated by 4 to 5 hours for 5 d/wk over 3 weeks. Two rats were excluded because they failed to reach the criterion (MacLellan et al., 2002) of 9 pellets (45 mg each) per forelimb on 3 consecutive days.

Tray Task

Rats were placed in the tray task for 30 min/d over 5 days before surgery. This apparatus is 19 cm wide X 27 cm long X 25 cm high and has 2-mm-wide vertical bars spaced 1 cm apart in the front through which rats reach for food (20- to 40-mg pellets) placed in a tray (4 cm wide and 5 mm deep; Whishaw, 2000). The rats' preferred paw was determined in this test.

Limb Use Asymmetry Test

Rats spontaneously explored (e.g., by touching the wall) a transparent cylinder (45 cm in height by 33 cm in diameter) during one 5-minute session for each of 2 days before surgery (MacLellan et al., 2002).

Horizontal Ladder Test

Rats walked across the ladder (1.0 m long) 3 times each day for 2 consecutive days before surgery (MacLellan et al., 2002; Metz and Whishaw, 2002). The ladder had randomly spaced rungs (1 to 3 cm).

Surgery

Rats were anesthetized with isoflourane (1.5% to 2% maintenance in 70% N_2O, 30% O_2), and a small hole was drilled in the skull (0.2 mm anterior and 3.0 mm lateral to bregma) contralateral to the preferred paw. A 28-gauge needle was inserted into the dorsal striatum (5.5 mm ventral to surface of the skull), and either 0.7 μL of sterile saline (SHAM group) or saline containing 0.14 U of bacterial collagenase (ICH group; type IV-S, Sigma Chemical Co) was infused (Rosenberg et al., 1990; Del Bigio et al., 1996; MacLellan et al., 2002). The needle was removed, and the craniotomy was sealed with a screw. Marcaine was infiltrated into the wound, which was then closed. Rectal

temperature was maintained between 36.0°C and 37.0°C during surgery with the use of a heating pad. Two rats were excluded because of surgical error.

Rehabilitation Therapies

One group each of SHAM and ICH rats received no therapy. Other groups received 7 days of rehabilitation beginning 1 week after surgery. Group assignment was random. Treated SHAM and ICH rats received either (1) CIMT alone (in standard home cage); (2) a daily exercise (EX) regimen; or (3) both (CIMT+EX). The CIMT involved restraining the forelimb ipsilateral to the lesion via a sleeveless jacket (model RJ02, Lomir Biomedical, Inc) that wrapped around the upper torso of the rat and attached to a metal wrist bracelet. The jacket was continuously worn for 7 days, while the bracelet restraint was used between 8 AM and 4 PM, mimicking human therapy (Wolf et al., 1989; Miltner et al., 1999). Rats had full range of motion when the bracelet was not used. The EX program lasted 1 h/d for 7 days. Exercises included 30 minutes in the tray task and 10 minutes in each of the cylinder, ladder, and running wheel tasks. Rats were required to walk across the ladder a minimum of 3 times and to run 10 m in the wheel. Rats were not exposed to the running wheel previously because it was not necessary.

Assessment of Recovery

Limb Use Asymmetry Test

Twenty-eight days after surgery, all rats were videotaped for 5 minutes in the cylinder. Forelimb use for initiating a rearing movement, for wall exploration, and for landing was analyzed (Tillerson et al., 2001; MacLellan et al., 2002; Metz and Whishaw, 2002). A "push-off" is the independent use of either forelimb or simultaneous use of both on rearing. "Wall exploration" is the initial placement of a forelimb on the wall and

contact during lateral movements across it. Simultaneous use on the wall includes lateral walking movements, in which limbs are used alternatively. A "landing" is the use of either forelimb or simultaneous use of both to land after rearing. Testing later was not done to limit the already extensive amount of behavioural analyses.

Horizontal Ladder Test

Twenty-eight days after surgery, rats were videotaped crossing the ladder 3 times. The percentage of footfalls (slips through the bars were summed across 3 trials) with each paw while traversing the ladder was analyzed.

Staircase Testing

All rats were food deprived (to 85%) 2 days before testing, which began 55 days after surgery. Rats were tested twice per day over 5 days (MacLellan et al., 2002). The number of pellets consumed per side, expressed as a percentage of baseline (average of last 10 training trials, i.e., an asymptotic performance), was analyzed. The staircase test was, a priori, our primary end point because it was meant to provide an unbiased assessment of recovery. Accordingly, this test was not used during rehabilitation so that we could determine whether the effects of rehabilitation on other tasks transferred to the staircase and whether those effects were persistent.

Elevated Body Swing Test

Sixty days after surgery, rats were tested on the elevated body swing test (EBST; Borlongan et al., 1995), which involved recording the direction of rotation after rats were suspended by the tail (30 trials). Rats normally turn ipsilateral to the lesioned hemisphere. Experimenters were blind to treatment identity, as with all tests.

Histology

Rats were killed 60 days after surgery with sodium pentobarbital (80 mg/kg) and perfused with saline and then 10% formalin. Brains were processed, and 40-μm (every 600 μm) coronal sections were taken with a cryostat and stained with cresyl violet (MacLellan et al., 2002). The volume of tissue lost was determined with the use of Scion Image J 4.0 (Scion Corporation) and expressed as:

(1) Volume of Tissue Lost = [Remaining Volume of Normal Hemisphere] – [Remaining Volume of Injured Hemisphere] and;

(2) Volume of Hemisphere = [Average Area of Complete Coronal Section of Hemisphere (Excluding Area of Ventricle and Area of Damage)] x [Interval Between Sections] x [Total Number of Sections]

Statistical Analysis

Data were analyzed with multiple factor (CIMT versus no CIMT, EX versus no EX, test trial) ANOVAs with subsequent simple effects and planned comparisons. In all cases the SHAM groups were combined (see Results), and thus comparisons with the combined SHAM group were made within a 1-way (e.g., volume data) or 2-way (staircase test data; between/within design) ANOVA (Keppel, 1982) or a *t* test that did not assume equality of error variances in cases of a significant Levene's test.

Results

Horizontal Ladder Test

Ipsilateral and contralateral fall rates were not different among SHAM groups (P 0.115). The percentage of falls with the contralateral forelimb was significantly (P 0.001) greater in ICH, ICH+EX, and ICH+CIMT groups than in the combined SHAM group (Figure 7.2). Whereas neither single therapy significantly lessened the ICH-

induced error rate (P 0.430), the combination treatment did ($P < 0.05$ versus untreated ICH; $P = 0.059$ versus SHAM). The ipsilateral forelimb fall rate in ICH groups did not differ statistically from the SHAM group ($P = 0.741$).

Cylinder Test

There were no significant group main effects in the percentage of simultaneous movements for push-off, landing, or wall contacts (P 0.082). Therefore, the percent contralateral use was analyzed [(number of contacts with contralateral limb/ ipsilateral + contralateral limb use) X 100]. Rehabilitation treatments did not affect SHAM groups (P 0.380), and data were combined. All ICH groups displayed an asymmetry favoring use of the ipsilateral limb (see Figure 7.3 for statistics). Because of high variability, only the ICH+CIMT+EX group was significantly better than the ICH group, but only for landing.

Staircase Test

All groups performed similarly during training (data not shown). The SHAM groups did not differ significantly during testing (percent baseline success) with the ipsilateral or contralateral limbs (P 0.158 for group main effects and group interaction). The untreated ICH group was impaired with the ipsilateral (P=0.041) and contralateral (P<0.001) forelimbs (Figure 7.4), although to a lesser extent with the ipsilateral limb. Neither the ICH+EX nor the ICH+CIMT group was significantly better than the untreated ICH group in terms of ipsilateral (P 0.599) and contralateral reaching success rate (P 0.318). The ICH+CIMT+EX group was significantly better (versus single or no therapies) with the contralateral (P<0.01) but not ipsilateral limb (P 0.106). This group was not significantly different than the SHAM group with either limb (P 0.547).

The first 5 minutes of the second last staircase session was videotaped to determine the number of reaches and success rate for those reaches (contralateral limb data shown). Although the group effect was not significant (P=0.164), there were trends toward less reaching in the ICH (mean ± SD reaches in 5 minutes = 31.2 ± 22.9), ICH+EX (32.0 ± 32.2), and ICH+CIMT groups (33.9 ± 23.1) compared with SHAM (48.6 ± 25.9), whereas the ICH+CIMT+EX group (50.1 ± 18.2) was similar to the SHAM group. The ICH (11.0 ± 12.8%), ICH+EX (9.2 ± 8.9%), and ICH+CIMT groups (5.5 ± 6.9%) had a lower success rate than SHAM (25.1 ± 14.9%; P 0.004). The ICH+CIMT+EX group (17.3 ± 10.3%) performed better than ICH rats, but this was not significant (P>0.10 versus ICH; P=0.091 versus SHAM).

Elevated Body Swing Test

The turning bias (percent toward lesioned side) results duringthe EBST did not statistically differentiate among SHAM or ICH groups with or without EX or CIMT treatments and did not reveal significant differences among lesioned and SHAM groups (P 0.065; SHAM averaged groups, -47.5%; untreated ICH rats, -64.2%; ICH+EX, -62.4%; ICH+CIMT, -55.2%; ICH+CIMT+EX, -49.1%).

Volume of Tissue Lost

Sham groups were not damaged (0.4 mm^3 of tissue lost ± 1.4 S.D.). There were significant differences among ICH groups (Figure 7.5), with a significant main effect for EX treatment (P = 0.049) and nonsignificant effects for CIMT treatment (P = 0.578) and the interaction (P = 0.736). Notably, the ICH+CIMT+EX and ICH+EX groups had a 28% and 18% smaller volume of tissue lost, respectively, than the untreated ICH group. A significant Levene's test for homogeneity of variances was found (P = 0.007). Thus,

additional *t* tests using separate or pooled error variances were also used depending on the outcome of each Levene's test. With this analysis, only the ICH+CIMT+EX group had a statistically smaller volume of tissue lost ($P = 0.028$).

Each behavioural test (e.g., contralateral limb in staircase test; $r = -0.609$, $P < 0.001$) significantly predicted the volume of tissue lost (all groups entered in analysis). A forward multiple linear regression of the average performance in the cylinder (percent contralateral touches), ladder (percent contralateral falls), EBST (percent ipsilateral turning), and staircase (percent success with contralateral limb) yielded an $r = 0.810$ ($P < 0.001$) for predicting the volume of tissue lost.

The reduction in the volume of lost tissue in the ICH+CIMT+EX group does not easily account for improved recovery in that group because the behavioural tests did not significantly correlate with volume in that group (e.g., average percent success with contralateral limb in staircase test; $r = 0.388$, $P = 0.238$). This is in contrast to the significant correlations within the ICH group (e.g., staircase data versus lesion volume; $r = -0.674$, $P = 0.023$), in which more direct relationships are expected.

Discussion

One week of combined CIMT and EX therapy significantly facilitated motor recovery on a number of behavioural tests after ICH in rats, whereas neither therapy alone was of as substantial benefit. This underscores the need for daily EX in combination with CIMT, as done clinically. Clinical studies show that CIMT, corresponding to our CIMT+EX treatment, successfully transfers improvement from the clinic to a real-life setting (Miltner et al., 1999; Taub and Morris, 2001). Similarly, rats were substantially better on the staircase test even though they were not rehabilitated on it

and testing started 6 weeks after the end of rehabilitation. Surprisingly, rats receiving combined therapy had significantly smaller volume of tissue lost, although this alone did not easily account for the improved recovery. While these results suggest that this therapy will benefit humans who suffer an ICH, further experimental studies examining the length, quality, and daily quantity of rehabilitation exercise and the insult severity are warranted given the findings that CIMT administered immediately after ischemic brain injury worsens outcome (Bland et al., 2000; unpublished data).

While the absence of a detrimental effect in this study is likely due to the greater intervention delay (Humm et al., 1998), it might have also resulted from using a different type of brain lesion (hemorrhagic versus occlusive) and/or a less demanding rehabilitation regimen (e.g., 8 h/d of limb restraint with bracelet and cotton jacket versus plaster of Paris cast). Thus, it is possible that immediate CIMT or the use of a 24-hour restraint method would aggravate an ICH. Our method of rehabilitation offers a definitive advantage over the casting method by allowing us to manipulate the duration of daily therapy. Another effective therapy is the combination of environmental enrichment and task-specific training, which has been shown to improve functional recovery when initiated 15 days after stroke in rats without affecting infarct size (Biernaskie and Corbett, 2001). Enrichment combined with CIMT and EX therapies might prove maximally beneficial.

We did not anticipate that rehabilitation therapy would lessen the apparent volume of tissue lost. However, this is not surprising given that exercise diminishes cell death and functional deficits from multiple types of brain insults. For instance, treadmill running (1 km/d) for weeks before, or immediately after, neurotoxic (demoic acid, 3-

acetylpyridine) and neurodegenerative insults (inherited Purkinje cell degeneration) is neuroprotective (Carro et al., 2001). Additionally, immediate forced use of the impaired forelimb ameliorates neurochemical injury and behavioural deficits in the 6-hydroxydopamine model (Tillerson et al., 2001). Currently, a cytoprotective effect was observed with rehabilitation delayed for 1 week after ICH. This is likely due to a reduction in atrophy and transneuronal degeneration because the rapid progression of the primary hemorrhagic injury in this model (Del Bigio et al., 1996) would make a direct neuroprotective effect unlikely. The reduction in injury may have occurred as a result of exercise-induced elevations in growth factors (e.g., insulin-like growth factor Carro et al., 2001) and exercise-induced angiogenesis (Kleim et al., 2002a). Further studies are examining some of these possible mechanisms. While a reduction in lesion volume would be expected to result in fewer behavioural deficits, it is unlikely to account solely for the marked improvements in the ICH+CIMT+EX group (e.g., poor correlation between volume and behaviour within the ICH+CIMT+EX group). Functional recovery results from many factors including, but not limited to, overcoming learned non-use, cortical (Nudo et al., 1996) and subcortical reorganization, synaptogenesis, and dendritic growth (Jones et al., 1999; Biernaskie and Corbett, 2001; Kleim et al., 2002b). The latter effects, if localized ipsilateral to the lesion, may contribute somewhat to the apparent reduction in the volume of lesion in the ICH+CIMT+EX group.

The superior performance of the ICH+CIMT+EX over the ICH+EX group in the staircase test is probably due to the fact that rats in the ICH+CIMT+EX group were forced to use their contralateral limb to obtain food in the tray task during rehabilitation, whereas the ICH+EX group likely used their ipsilateral limb even though it was not their

originally preferred paw. We previously observed that rats with their preferred forelimbs impaired (via Botulinum A toxin injections) learned to use their nondominant limb until the toxin became inactive (unpublished data). Accordingly, the advantage of the CIMT therapy is that it forces the rats to use their contralateral limb during specific rehabilitation exercises and at other times (e.g., grooming), although the contribution of each is unknown. The most impressive benefits were observed in the staircase and ladder tests probably because rats participated actively in the tray and ladder tests during rehabilitation. While the ICH+CIMT+EX group performed similar to the SHAM group with both limbs in the staircase test, only the contralateral limb results were significantly better than those in untreated ICH rats. This is likely due to a smaller ipsilateral deficit in untreated ICH rats because there was no residual deficit with the ipsilateral limb of the ICH+CIMT+EX rats. The less impressive recovery in the cylinder test may be due to rats becoming habituated and participating less. Additionally, the EBST and the cylinder test were less useful in detecting group differences because of substantial intragroup variability. Nonetheless, all of the behavioural tests used significantly correlated with the volume of injury (with all animals included).

Of many putative neuroprotectants administered after striatal ICH in rats (eg, hypothermia (MacLellan et al., 2002), hematoma aspiration (Altumbabic et al., 1998), NXY-059 (Peeling et al., 2001), anti-inflammatory therapies (Del Bigio et al., 1999), none have provided benefit in the staircase test, in contrast to the substantial and persistent recovery presently observed with CIMT+EX therapy. Nonetheless, caution must be exercised in interpreting the observed recovery since rats may have used compensatory strategies (Whishaw et al., 1997). For example, some groups had a lower

percent reaching success rate in the staircase test. Further studies should examine whether CIMT+EX therapy promotes true recovery of function.

In summary, CIMT+EX therapy significantly and chronically improved motor deficits after ICH in rats. Clinical investigations are warranted in humans who suffer intrastriatal hemorrhagic stroke. Further experimental studies are also needed to elucidate the mechanisms underlying the facilitated recovery and reduction in the volume of tissue lost and the factors that promote maximal recovery (e.g., duration of therapy).

Table 7.1

See Figure 7.1 for time line. Rats were randomly assigned up to desired group size of at least in SHAM groups and at least 8 in ICH groups.

Group Sizes

	CIMT		No CIMT	
	EX	No EX	EX	No EX
SHAM	6	6	5	6
ICH	11	9	9	11
	ICH+CIMT+EX	ICH+CIMT	ICH+EX	ICH

Figure 7.1

Group treatments (days relative to surgery). The table shows group sizes.

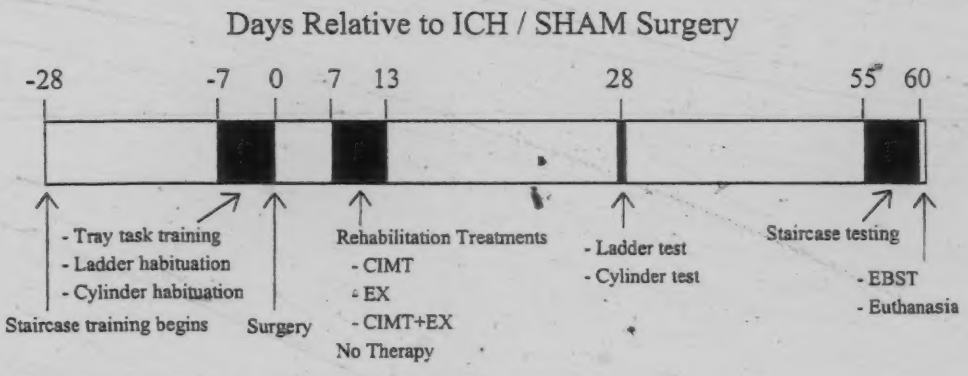

Days Relative to ICH / SHAM Surgery
-28
-7
0
7
13
28
55
60
- Tray task training
- Ladder habituation
- Cylinder habituation
Staircase training begins
Surgery
Rehabilitation Treatments
- CIMT
- EX
- CIMT+EX
No Therapy
- Ladder test
- Cylinder test
Staircase testing
- EBST
- Euthanasia

Figure 7.2

Number of falls (mean ± S.D.) with ipsilateral and contralateral forelimbs while crossing the ladder on day 28 after surgery. *P < 0.05 versus SHAM; †P < 0.05 versus untreated ICH.

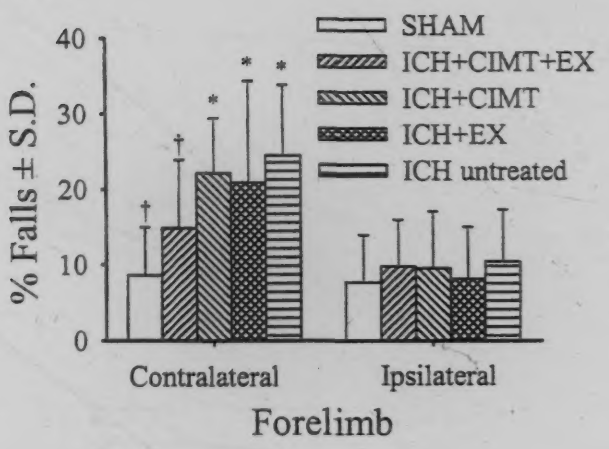

SHAM
ICH+CIMT+EX
ICH+CIMT
ICH+EX
ICH untreated
% Falls ± S.D.
40
30
20
10
0
†
†
*
*
*
Contralateral
Ipsilateral
Forelimb

Figure 7.3

Spontaneous paw use (mean ± S.D. percent use of contralateral forelimb) in the cylinder on day 28 after surgery. *P < 0.05 versus SHAM; †P < 0.05 versus untreated ICH.

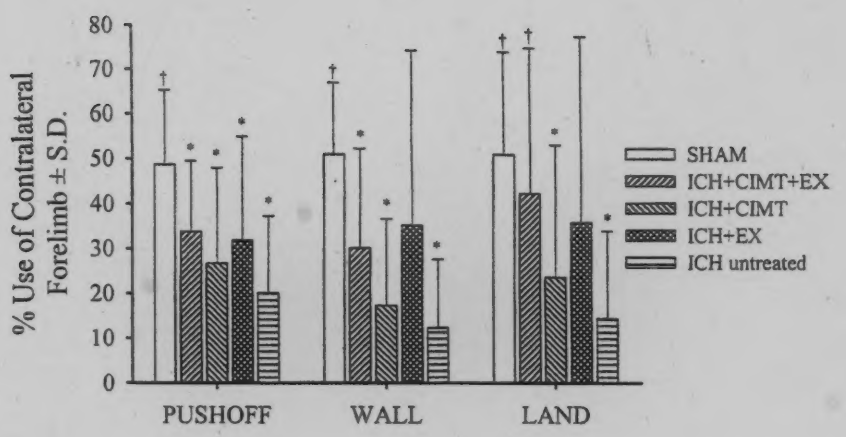

% Use of Contralateral Forelimb ± S.D.
80
70
60
50
40
30
20
10
0
PUSHOFF
WALL
LAND
SHAM
ICH+CIMT+EX
ICH+CIMT
ICH+EX
ICH untreated

Figure 7.4

Average percent reaching success in the staircase with ipsilateral and contralateral forelimbs. Greater contralateral deficits were found than with the ipsilateral forelimb. The ICH, ICH+CIMT, and ICH+EX groups were significantly impaired compared with SHAM. The ICH+CIMT+EX group had near-total recovery (see Results for statistics).

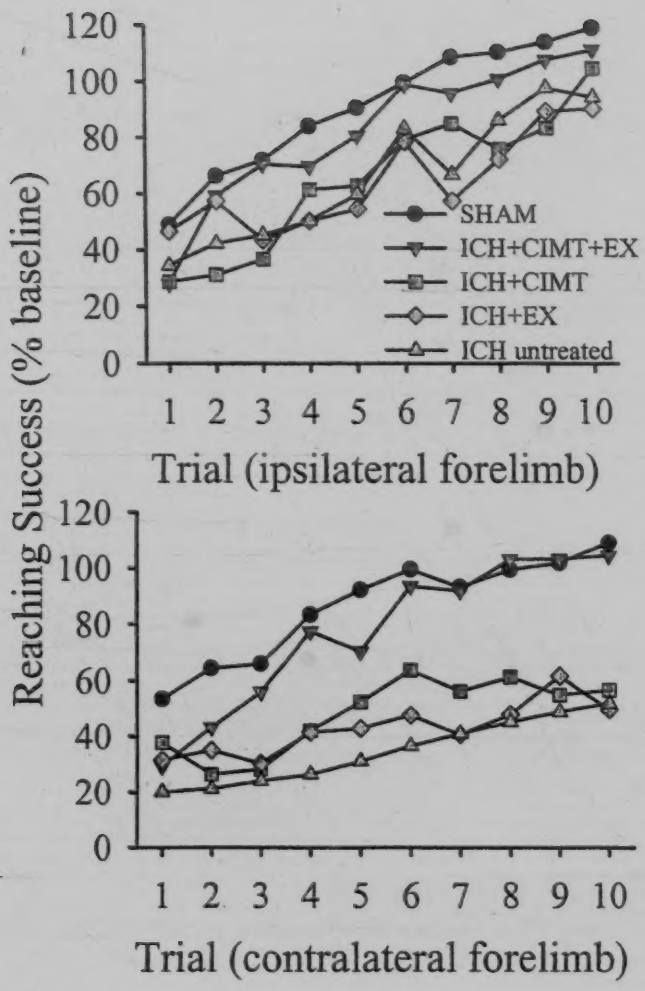
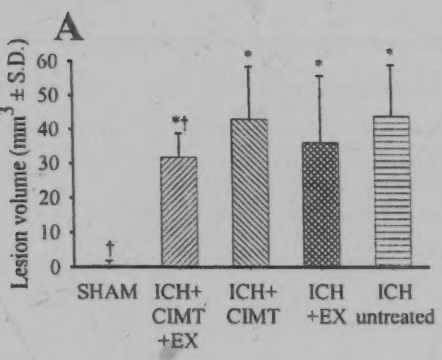

Reaching Success (% baseline)
120
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10
Trial (ipsilateral forelimb)
SHAM
ICH+CIMT+EX
ICH+CIMT
ICH+EX
ICH untreated
120
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10
Trial (contralateral forelimb)

Figure 7.5

Volume of tissue lost at 60 days after ICH/SHAM surgery (A). The calculation includes the cavity and atrophy (eg, ventriculomegaly; photomicrograph represents a typical lesion [B]). Injury was less in the ICH+EX group and significantly less in the ICH+CIMT+EX group. *$P < 0.05$ versus SHAM; †$P < 0.05$ versus untreated ICH.


A
Lesion volume (mm^3 ± S.D.)
60
50
40
30
20
10
0
SHAM
ICH+ CIMT +EX
ICH+ CIMT
ICH +EX
ICH untreated

B

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Chapter 8

General Discussion

The main focus of this thesis was to examine the vulnerability of neurons damaged by ischemic and hemorrhagic injury. First and foremost, it is hoped that the experiments herein will not only advance our knowledge of the neural mechanisms underlying tissue vulnerability after brain injury, but also more importantly promote recovery of both neurological and functional outcome in the human stroke patient. Despite overwhelming amounts of research being done to understand the mechanisms underlying stroke-induced neuronal injury in animal models, a promising drug that protects neurons from the various types of insult remains unavailable in the clinic. Following stroke, neural tissue surrounding injury suffers one of two fates. Cells will both survive injury and reorganize to compensate for the loss of function (i.e., cortical plasticity), or become subjected to secondary degenerative events. Current research is attempting to promote the former, either through preventing neuronal degeneration or encouraging survival of neurons. This thesis illustrates that factors such as behavioural demand and temperature fluctuations can profoundly affect neuronal survival following injury.

Constraint-induced movement therapy (CIMT) provides lasting functional improvement to stroke patients suffering from hemiplegia, even when applied months after stroke (Ostendorf and Wolf, 1981; Wolf et al., 1989; Taub et al., 1999; van der Lee et al., 1999a; van der Lee et al., 1999b; Dromerick et al., 2000; Taub and Morris, 2001). It remains unknown however, whether an optimal therapeutic window exists earlier after injury in which CIMT could build upon lesion-induced plasticity. For instance, in some cases following stroke, patients spontaneously recover, suggesting the brain has the potential to reorganize its own functioning. Unfortunately, not all stroke survivors

experience this remarkable recovery. The type, and intensity of rehabilitation a patient receives can therefore drastically alter outcome. Clinically, rehabilitation strategies such as CIMT are based upon the common theory of "use-it-or-lose-it"(Schallert et al., 1997). In the normal brain, evidence from several studies indicates that the size of the cortical representation of a body part in the human depends on the use of that body part (Schallert et al., 1997; Buonomano and Merzenich, 1998). This theory developed due to the observation that cortical representation of the fingers were larger in left handed string players than in control subjects (Elbert et al., 1995). *Injury*-induced cortical plasticity can also occur as demonstrated in monkeys following digit amputation (Elbert et al., 1995). Moreover, use-dependent reorganization of cortical maps occurs following injury as a result of rehabilitation (e.g., CIMT) in stroke patients (Liepert et al., 1998). Perhaps therefore, it is possible that rehabilitation initiated soon after injury could act in combination with ongoing injury-induced reorganization.

In the rodent, we found CIMT provided functional recovery, as well as decreased lesion volume after intrastriatal hemorrhagic stroke. This is the first behavioural treatment showing improved functional outcome after hemorrhagic stroke on skilled reaching, walking and limb-use asymmetry tests (DeBow et al., 2003). Treated rats also had decreased injury volumes compared to rats that did not use their impaired limb (DeBow et al., 2003). Similarly, early forced use of the impaired limb after unilateral 6-hydroxydopamine lesions also provided functional recovery and was neuroprotective (Tillerson et al., 2001). Following proximal middle cerebral artery occlusion, early *disuse* of the impaired limb resulted in detrimental effects on functional outcome, without aggravation neuronal damage (Bland et al., 2001). These data conflict with previous

evidence showing deleterious effects of CIMT after distal middle cerebral artery occlusion (Bland et al., 2000) and unilateral electrolytic lesions of the sensorimotor cortex (Kozlowski et al., 1996; Humm et al., 1998). Perhaps the discrepancy in these results is due to the location of injury, as the effect of CIMT differs depending on whether cortical or sub-cortical structures are damaged. The CIMT-induced exacerbation of injury occurs following cortical injuries, but not following sub-cortical injury. Taken together, these studies along with our data in the intrastriatal hemorrhagic model (DeBow et al., 2003), suggest there may be a difference in the response to demands of CIMT on neurons in the cortex and the striatum. Perhaps a better understanding of striatal injury could provide insight into new approaches to preventing CIMT-induced exacerbation in the cortex.

Evidence suggests that approximately 20% to 25% of patients with chronic ischemic injury may benefit from CIMT (Blanton and Wolf, 1999). However, shortened lengths of rehabilitation stays on stroke wards have forced clinicians to use therapies that do not require a lot of time while continuing to provide functional benefit. Moreover, therapy must therefore also be initiated as soon as possible following injury. As previously discussed, early administration of CIMT in the rat not only worsens neurological outcome, but it also slows functional recovery (Kozlowski et al., 1 996; Humm et al., 1998; Bland et al., 2000). This thesis further supports these data. Contrary to the beneficial effect of CIMT following intrastriatal hemorrhage, we found CIMT applied following cortical devascularization potentiated neurological outcome (unpublished data). These results suggest that although use-dependent plasticity is possible following sub-cortical injury, the penumbral region surrounding injury in the

cerebral cortex may be vulnerable to behavioural pressure for some time following injury.

Alternatively, we found CIMT produced significant increases in localized brain temperature (unpublished data) in the tissue surrounding injury. Evidence suggests hyperthermia (e.g., fever) during, or after cerebral injury is deleterious to vulnerable brain regions (Ginsberg and Busto, 1998). Thus, it is likely hyperthermia plays a significant role in CIMT-induced exacerbation of injury when therapy is applied early in the post-lesion period. Since animal research has provided ample evidence suggesting *early* CIMT is detrimental, it is possible clinicians will not use CIMT. Clinically, traditional rehabilitation focuses on compensatory strategies to promote functional recovery. However, this may cause more harm as traditional forms of rehabilitation often result in lasting impairments of the hemiplegic limb, and teaches patients to not use their impaired limb, a phenomenon known as "learned non-use" (Ostendorf and Wolf, 1981; Wolf et al., 1989; van der Lee et al., 1999b; Dromerick et al., 2000). Therefore, studies characterizing the source and role of hyperthermia in early CIMT are critical. For instance, how long after injury must CIMT be delayed to prevent the deleterious effects observed in the rodent? Moreover, are other factors other than behavioural demand and hyperthermia capable of damaging vulnerable neurons during this critical period post-lesion?

Using the gerbil model of global ischemia, the resiliency of hippocampal CA1 neurons previously treated with cytoprotective agents to secondary stress was examined. Hypothermia has been deemed the "gold-standard" neuroprotective therapy after stroke. Both intra-ischemic (Dietrich et al., 1996), and prolonged post-ischemic hypothermia substantially attenuate ischemia-induced CA1 neuronal death (Colbourne and Corbett,

1994; Colbourne and Corbett, 1995; Colbourne et al., 1999; Hickey et al., 2000). However, although hypothermia treated cells survive injury, they remain susceptible to secondary injury (DeBow and Colbourne, 2003). We found CA1 hippocampal neurons were significantly protected by postischemic hypothermia even after a 12 hour delay (DeBow and Colbourne, 2003). Secondary injury, which consisted of sub-lethal transient ischemic attacks were sufficient however, to undo the protection hypothermia provided. This evidence is supported by previous findings involving neuronal protection afforded by ischemic preconditioning (EE; Farrell et al., 2001). In that study, post-ischemic environmental enrichment reduced neuroprotection achieved earlier with ischemic preconditioning. Thus, although neuroprotection is possible following ischemia, neurons remain susceptible to secondary injury.

Although behavioural experience can enhance neuronal growth after brain injury in remote, intact brain areas, rehabilitation strategies focused specifically on recovery of injured function may actually be able to prevent axonal loss in the damaged cortex. Experience promotes growth of new neurons in the damaged hemisphere (Kolb et al., 1998; Kolb and Whishaw, 1998). Thus, perhaps "use-it-but-don't-overuse-it" may be better suited for recovery of vulnerable tissue surrounding injury.

Limitations

Transferability of Rodent Data to the Clinic

Stroke is one of the oldest recognized diseases, but remains one of the least understood (Elkind, 2003). Numerous prospects for improved neuroprotection in the rodent have failed to protect injured tissue in the human. Despite these disappointing results however, researchers remain optimistic that future clinical trials will yield positive

results. In fact, failure to provide benefit has caused researchers to question a number of aspects of experimental procedures. For instance, neuroprotective agents are often given before, during or immediately after injury. Clinically however, this is extremely difficult to achieve. Thrombolytic agents, such a tissue plasminogen activator (t-PA), provide benefit to patients only if applied within 3 hours after stroke (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995). However, when given after the therapeutic window, t-PA increases the risk of hemorrhagic transformation (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995). Age is one of the most important risk factors for stroke (Wolf et al., 1992). However, the majority of experimental studies assessing neuroprotective agents fail to assess injury outcome in aged animals (DeBow et al., in press). Similarly, we found female rodents are rarely assessed in neuroprotective studies despite the fact that women stroke victims significantly outnumber men (Elkind, 2003). We also report female rodents are rarely assessed in neuroprotective studies, unless the effects estrogen on the cytoprotectant is being examined directly (DeBow et al., in press). Researchers are just recently beginning to change their research strategies in accordance with the many limitations of animal research. In the past, safety and side effects caused by large doses of a neuroprotective agent had previously been ignored. However, dosing regimens have since improved substantially due to the fact that human dosing was often plagued by side effects (Davis et al., 2000). In summary, there are a number of limitations affecting animal research that need to be addressed and corrected in order for progress to continue.

It has been argued that the rodent model is not a useful tool when examining human injury, as too many species differences exist. However, in a recent survey

(DeBow et al., in press), we found bilateral common carotid artery occlusion in the gerbil to be the most common global model of ischemia (DeBow et al., in press). This may be, in part, due to the short and simple surgical method. Despite this, controversy exists however, regarding whether or not results from the gerbil can be generalized to the rat and human. One major anatomical difference exists in the gerbil in that they do not have a complete circle of Willis. Therefore, rat and human injury differ from the gerbil due to collateral blood flow from posterior communication arteries. It is argued that vital difference may profoundly affect the ability of researchers to transfer results from the gerbil to humans. It is worth noting however, that ischemic injury in the gerbil closely reflects that observed in the human brain following cardiac arrest (Zola-Morgan et al., 1986). In spite of disappointing results of neuroprotection strategies over the past two decades, investigations into treatment of stroke in both the rodent and human have only become more vigorous. Importantly, a number of methodological improvements, including the use of aged and female animals, need to be addressed in the laboratory.

Future Studies

This thesis has presented substantial evidence that temperature fluctuation after stroke can substantially impact outcome. It is therefore crucial for future studies to determine *how* temperature is causing cell death, and more importantly preventing recovery after stroke. We concluded hyperthermia early after cortical injury was, at least in part, responsible for rehabilitation-induced exacerbation of injury, however the source of this fever remains unclear. There are two possibilities. The first is a stress-induced fever due to the casting procedure itself. Restraint of the whole animal can severely perturb the thermoregulatory system, and can alter the animal response to physical

stimuli (Gordon, 1990). If CIMT were causing a stress induced fever in susceptible brain regions, perhaps preventing the potential stress induced response (e.g., anxiolytic agents) would elucidate the source of CIMT-induced hyperthermia. The second possibility is that hyperthermia in localized brain regions occurs during rehabilitation due to excessive behavioural demand (i.e., over-stimulation) of vulnerable tissue. Thus, perhaps forced use of the impaired limb increases metabolic functions in the tissue surrounding the injury, thereby increasing temperature. Difficulty in preventing activity-dependent rises in temperature occur however, as inhibiting movement of the impaired limb (the same limb the animal is forced to use) proves difficult. Perhaps glutamate receptor antagonists (e.g., MK-801) could be used to prevent over-activity of neurons in the injured cortex in an attempt to determine if localized hyperthermia would still occur during CIMT. If so, this would suggest hyperthermic responses to CIMT are not due to increased neuronal activity. Nonetheless, future studies examining and elucidating the source of CIMT-induced hyperthermia would profoundly alter our understanding of post-ischemic temperature changes.

There are numerous components involved in constraint-induced movement therapy. In humans, CIMT involves restraining movement of the unimpaired limb and shaping movements of the unpaired limb for many hours a day for two or three consecutive weeks (Taub et al., 1993). In rodents, we found CIMT provided functional improvements on a number of behavioural tasks. More importantly, we found CIMT rats had significantly smaller lesions. There were two main components to the CIMT regimen we used. First, the impaired limb is restrained for 8 hours a day. Second, CIMT rats underwent intensive exercise regimes while their limb was restrained. Exercise alone

diminishes cell death and functional deficits after neurotoxic (demoic acid, 3-acetylpyridine) and neurodegenerative insults (inherited Purkinje cell degeneration; (Carro et al., 2001). It is therefore likely that the exercise component itself provides a substantial amount of the recovery observed after intrastriatal hemorrhage. Future studies are needed to determine the role of exercise in CIMT-induced recovery. Would exercise alone provide neurological protection similar to that observed in CIMT rats? Presumably, skilled training of the impaired limb is required to provide functional recovery on specific behavioural tasks, but perhaps exercise alone is sufficient to reduce lesion size.

Summary

These data conclusively show that injured brain tissue is vulnerable to further injury following stroke. Moreover, that secondary injury may be caused by factors previously known to cause injury (e.g., hyperthermia), or factors that are thought to provide recovery (e.g., rehabilitation). Such findings suggest care and attention to these potential deleterious factors is warranted in future studies assessing neuroprotective agents, as well as rehabilitative strategies, on neurological outcome. Brain temperature fluctuations in the brain and body, perhaps due to the cytoprotective agent itself, must be closely monitored and controlled. Moreover, rehabilitative strategies, as well as intense behavioural tasks should be administered with caution to only those patients physically capable of performing them.

These data also show that neurological and functional recovery is possible following stroke. Postischemic temperature afforded neuroprotection in gerbils prior to secondary injury. Further, for the first time, behavioural and histological recovery was afforded following intrastriatal hemorrhage. It is hoped that future studies focus on

combining these positive results and continue to promote further neuroprotection and recovery following various types of injury.

The experimental basis for neuroprotection, although plagued with numerous problems, is well founded. It is up to researchers from the bench to the bedside to overcome these issues and ensure that future clinical trials do not continue to suffer the same fate. Importantly, compounds selected for clinical trials must be selected on the basis extensive animal testing, assuming a number of previously determined criteria are met. Issues such as adequate and strict temperature regulation during and after ischemia, as well as the assessment of all compounds in aged animals must be demonstrated. Benefit should also be clearly illustrated in both male and female animals. Although the use of both genders as well as aged animals will likely increase costs of experimental research significantly, the benefit of discovery a useful neuroprotective agent more rapidly outweighs them. With these numerous potential improvements to experimental research needing to be addressed, there is hope research will overcome these issues and change the face of stroke as we know it.

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